Diabetic nephropathy

Pathology, genetics and carnosine metabolism

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General introduction

Diabetic nephropathy, a progressive kidney disease due to longstanding diabetes, is the leading cause of end stage renal disease in the Western world (1). However, not all patients with diabetes-mediated hyperglycemia will develop this disease (2;3;4). It appears that both environmental and genetic factors play a role in the development of diabetic nephropathy, making diabetic nephropathy a complex disease.

One of the aims of this thesis was to create a histopathological classification of diabetic nephropathy. Another goal was to create a systematic overview of genetic associations in diabetic nephropathy. Furthermore, we focused more on one of these genetic variants, the number of trinucleotide repeats coding for leucine in the *CNDP1* gene. Concerning this genetic variant, we examined the genotypic distribution in 3 ethnic groups. Additionally, we investigated whether the association between this genetic variant and diabetic nephropathy was sex-specific. Furthermore, the association between the *CNDP1* genotype and other progressive glomerular diseases was studied. Finally, as the *CNDP1* gene encodes for the enzyme carnosinase and carnosinase breaks down carnosine, which is known to have many protective capacities, we searched for determinants of carnosine levels.

The outline of this introduction is as follows. First, an introduction on diabetes, the kidney and diabetic nephropathy (part I) will be given, followed by an introduction on genetics in diabetic nephropathy (part II). Continuing with the *CNDP1* gene, the enzyme it codes for, carnosinase-1 and its substrate L-carnosine (part III).

Part I – Diabetes, the kidney and diabetic nephropathy

Diabetes

Diabetes mellitus is a metabolic disorder of multiple causes which is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism. The word diabetes originates from two Greek words; 'dia' (=διαα) means 'through' and 'bainein' (=βαινειν) means 'to pass'. The word mellitus comes from Latin, meaning 'honey sweet'. Therefore, diabetes mellitus could be translated as 'honey sweet passage'.

The two most common types of diabetes mellitus are type 1 and type 2 diabetes. Type 1 diabetes, accounting for 5-10% of cases of diabetes in populations of European origin, is associated with primary beta cell failure, mostly a result of autoimmune

destruction. Type 2 diabetes, the most common type in all populations, is characterized by high glucose in the context of insulin resistance and relative insulin deficiency. The number of type 2 diabetes patients has rapidly increased in the past few decades and is still rising further. In 2002, the number of diabetes patients worldwide was estimated at 173 million and has been predicted to increase to 350 million in 2030 (1).

Approximately 5% of the diabetes patients have maturity onset diabetes of the young (MODY). MODY is caused by a genetic defect leading to pancreatic beta cell dysfunction. One percent of the diabetes patients have mitochondrial diabetes, due to defects in the mitochondria.

Long-term hyperglycemia can lead to severe complications later in life. These complications of diabetes are divided in macrovascular and microvascular complications. Macrovascular disease is mainly confined to cardiovascular disease. Microvascular complications occur mainly in the eyes, kidneys, peripheral lower limbs and nerves, resulting in diabetic retinopathy, diabetic nephropathy, diabetic foot and diabetic neuropathy respectively. In this thesis we will further focus on diabetic nephropathy due to type 1 and type 2 diabetes.

The kidney

To understand the pathology of diabetic nephropathy we first briefly describe the anatomy and function of the human kidney. The kidneys are the main excretory organs of the human body. They regulate the extracellular volume, water and salt balance, and acid-base homeostasis. Furthermore, kidneys are endocrine organs, producing hormones with a role in erythropoiesis, calcium metabolism and blood pressure regulation.

Each kidney weighs approximately 150 grams in adults, and is located in the retroperitoneum. A kidney has three major components: the cortex, the medulla and the collecting system. The cortex, the outer layer of the kidney, consists mainly of glomeruli and convoluted tubuli. Situated more to the centre is the medulla consisting of pyramidal structures of parallel arranged tubular structures with apical papillae. The bases of the pyramids are at the corticomedullary junction and the apices extend into the collecting system. In the collecting system the pre-urine goes from the minor calyces, which receive pre-urine from a medullary papilla, to the major calyces, pelvis and finally it enters the ureter.

The blood supply of each kidney is by a single renal artery originating from the abdominal aorta. The main renal artery branches form anterior and posterior divisions

at the hilus and divides further into the interlobar arteries which run between lobes. Interlobar arteries extend to the corticomedullary junction and give rise to arcuate arteries, which arch between cortex and medulla. Afferent arterioles branch off from the arcuate arteries, each directing to a single glomerulus. A glomerulus represents a spherical bag of capillary loops arranged in several lobules. The capillaries come together to exit the glomerulus through the efferent arteriole. In most nephrons, the efferent arterioles branch off to form another vascular bed, the peritubular capillaries, which surround the tubules.

A nephron is a functional unit of the kidney and consists of a glomerulus with attached tubuli. Each kidney has approximately one million nephrons. The glomerulus consists of 4 types of cells: the mesangial cell, endothelial cell, visceral epithelial cell (podocyte), and parietal epithelial cell. The mesangial cells are responsible for the production of the mesangial extracellular matrix. Mesangial cells have numerous functions including contraction, production of extracellular matrix, secretion of inflammatory and other active mediators and phagocytosis. Mesangial cells and their mesangial matrix together form the mesangium.

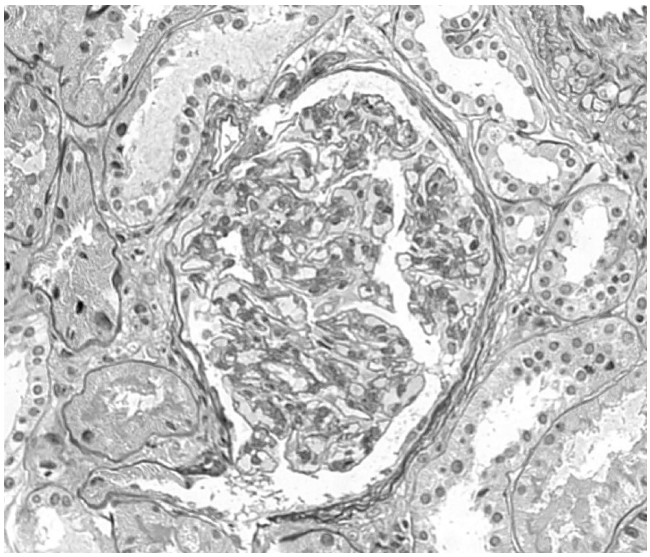

Figure 1. Histology of a normal glomerulus

The blood from the capillary network of the glomerulus is filtered into the tubuli. The filtration barrier of the glomerulus consists of endothelial cells, glomerular basement membrane and podocytes. Endothelial cells of the glomerulus are thin and have multiple fenestrae with a glycocalyx, extracellular polymeric material at the apical site of the endothelial cell. The glomerular basement membrane is a 300-350 nm thick network that surrounds the glomerular capillaries. The outer aspects of the glomerular capillaries are covered with podocytes. Each podocyte has a large body with small finger-like processes that interdigitate with similar structures from adjacent cells. These structures cover the glomerular capillaries forming slit pores. Blood is filtered through these slits, known as a slit diaphragm. After passing through the glomerular filtration barrier and leaving large and negatively charged molecules in the capillaries, the filtrated blood enters the Bowman's space which is aligned by parietal epithelial cells. The Bowman's space is continuous with the tubular system.

The remaining portion of the nephron consists of tubuli. First, the filtrated blood enters the proximal tubule, then enters the loop of Henle and finally the distal tubules. These structures concentrate the pre-urine and reabsorb essential molecules, but also secretion from blood to tubuli occurs. The tubuli are supported by the interstitium, which consists of thin connective tissue in peritubular and periarterial spaces.

Diabetic nephropathy

The UK prospective diabetes study (UKPDS) and diabetes control and complications trial (DCCT) have established an initiating role for hyperglycemia in developing microvascular complications such as diabetic nephropathy (2;5).

There are four main hypotheses about how hyperglycemia causes diabetic nephropathy:

1) increased polyol pathway flux,
2) increased advanced glycation end product formation,
3) activation of the protein kinase C isoforms,
4) increased hexosamine pathway flux.

Each of these hypotheses will be described in detail below:

1) Aldose reductase is the first enzyme in the polyol pathway and an oxidoreductase that catalyses the NADPH-dependent reduction of a wide variety of carbonyl compounds, such as glucose. Aldose reductase has a low affinity for glucose and in the normal situation the metabolism of glucose by this pathway is minimal. In a hyperglycemic environment, increased intracellular glucose results in its increased enzymatic conversion

to sorbitol, with concomitant decrease in NADPH. In the polyol pathway, sorbitol is oxidized to fructose by the enzyme sorbitol dehydrogenase with NAD^+ being reduced to NADH. The most accepted theory how the polyol pathway is involved in causing diabetic nephropathy is as follows. Reduction of glucose to sorbitol consumes NADPH. As NADPH is required for generating glutathione, and glutathione protects cells from oxidative stress, this could induce intracellular oxidative stress (6;7).

2) Advanced glycation end products (AGE) are irreversibly damaged proteins or lipids resulting from a chain of chemical reactions after an initial glycation reaction (8). AGE formation is increased in diabetes due to increased intracellular glucose. Intracellular and extracellular AGEs and its precursors damage cells by three mechanisms. First, intracellular proteins modified by AGEs have an altered function. Second, extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with the receptors for matrix proteins, such as integrins on cells. Third, plasma proteins modified by AGE precursors bind to AGE receptors on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production of reactive oxygen species.

3) Intracellular increase of glucose augments the synthesis of a molecule called diacylglycerol (DAG), which is a critical activating cofactor for the classic isoforms of protein kinase C (PKC), β, δ and α. PKC has an effect on expression of a variety of genes. Its overactivation leads to blood flow abnormalities, vascular permeability, capillary and vascular occlusion, pro-inflammatory gene expression and oxidative stress (9-11).

4) In the hexosamine pathway, fructose-6-phosphate is diverted from glycolysis leading to an increase in uridine diphosphate-*N*-acetylglucosamine. Overmodification by this glucosamine of serine and threonine residues leads to pathological changes in gene expression and protein function. Although a role for this relatively newly identified pathway in diabetic nephropathy is evident (12;13), the exact pathogenic mechanisms still need to be established.

Brownlee (14) hypothesized that these four pathways can be linked together by a common initiating factor: superoxide formation by the mitochondria. Increased hyperglycemia-derived electron donors from the tricarboxylic acid cycle (also known as the citric acid cycle), NADH and $FADH_2$, generate a high mitochondrial membrane potential, by pumping protons across the mitochondrial inner membrane. This inhibits electron transport, increasing the half-life of free radical intermediates of co-enzyme O, which reduce O_2 to superoxide. Hyperglycemic induced superoxide formation

by the mitochondria decreases GAPDH which converts glyceraldehyde-3-P into 1,3-diphosphoglycerate in the tricarboxylic acid cycle. As a result glyceraldehyde-3-P, which is a precursor for AGE and PKC, increases. This initiates AGE formation and PKC activation. Due to the reduced conversion by GAPDH, the molecules upstream the tricarboxylic acid cycle will also increase. These are fructose-6-P, activating the hexosamine pathway, and glucose itself, increasing the polyol pathway activity.

Early in the course of diabetes, abnormalities in blood flow and increased vascular permeability occur. In this stage, there is decreased activity of vasodilators, such as nitric oxide and increased activity of vasoconstrictors, such as angiotensin II and endothelin-1 and elaboration of permeability factors such as vascular endothelial growth factor (VEGF). Later, abnormalities in the extracellular matrix contribute to an irreversible increase in vascular permeability. With time, microvascular cell loss occurs, in part as result of apoptosis, and there is progressive capillary occlusion due to both extracellular matrix overproduction induced by growth factors such as transforming growth factor β (TGF-β) and deposition of hyaline material. Together, these changes lead to oedema, high blood pressure in the glomerulus and ischemia, finally leading to glomerulosclerosis.

The abovementioned mechanisms are not confined to diabetic nephropathy. They also play a role in macrovascular complications and other microvascular complications.

Clinical features

Clinically, the natural history of diabetic nephropathy is described to consist of five stages (15). The first stage is characterized by hyperfiltration and hypertrophy of the glomerulus, leading to higher glomerular filtration rate and renal enlargement. This stage is still reversible but is associated with an increased risk of developing more advanced diabetic nephropathy (16). Stage 2 develops silently over many years and is characterized by morphologic lesions without signs of clinical disease. Then diabetic nephropathy progresses from microalbuminuria (incipient diabetic nephropathy, stage 3) to macroalbuminuria (overt nephropathy, stage 4) and finally, end stage renal disease (advanced diabetic nephropathy). Microalbuminuria can still regress in contrast to the later stages. When high blood pressure in the stage 4 diabetic nephropathy (macroalbuminuria) is left untreated, renal function (GFR) declines, the mean fall rate being around 1 ml/min/month. Long-term antihypertensive treatment reduces the fall rate by about 60% and thus postpones end stage renal disease considerably (15). End stage renal disease refers a stage in which patients require dialysis or kidney transplantation, because the kidneys do not function anymore.

There is still debate whether diabetic nephropathy due to type 1 and type 2 diabetes can be considered the same disease. Two early studies showed a similar course in clinical diabetic nephropathy in type 1 and type 2 diabetes (17;18). However, several differences have been described between diabetic nephropathy in type 1 and type 2 diabetes. Diabetic nephropathy due to type 1 diabetes almost always coincides with diabetic retinopathy (19) in contrast to type 2 diabetes in which this parallel is less clear. However, the relationship between diabetic nephropathy and retinopathy in type 2 diabetes varies depending on the diabetes regimen (20). In patients treated with insulin, the relationship between nephropathy and the severity of retinopathy is similar to that in type 1 diabetes (21). Furthermore, some claim that diabetic nephropathy develops more often in type 1 diabetes than in type 2 diabetes. The difficulty in type 2 diabetes is that many die due to cardiovascular disease before reaching the clinical stage of diabetic nephropathy. If we look at Pima Indians who have type 2 diabetes relatively early in life and are known for their relatively low cardiovascular mortality risk, 65% of the diabetes type 2 patients will develop ESRD (22), suggesting that cardiovascular disease in Caucasians might influence the incidence rate of diabetic nephropathy due to type 2 diabetes.

For pathologists, diabetic nephropathy due to type 1 and type 2 diabetes appears to be undistinguishable (23;24). However, on the genetic level, there seem to be differences in genetic susceptibility between diabetic nephropathy due to type 1 and type 2 diabetes (25-29). This is suggestive for different mechanisms in type 1 and type 2 diabetes leading to similar histopathologic appearance of diabetic nephropathy.

Histopathology

Diabetic nephropathy causes pathological abnormalities in the glomerulus and tubulus extraglomerular vessels and interstitium. The first pathological sign of diabetic nephropathy is enlargement of the glomerulus which corresponds to the clinical stage of glomerular hyperfiltration (stage 1) (15). Additionally, glomerular basement thickening occurs in an early stage and is seen to be present after 2 years of diabetes duration (30). When the disease progresses the mesangium starts to expand, corresponding to the clinical stage of both micro- and macroalbuminuria.

Two forms of diabetic nephropathy are described; diffuse versus nodular glomerulosclerosis (31). This designation is primarily of descriptive value, because the distinctions do not have clear-cut clinical significance, although nodular glomerulosclerosis is more often described in severe cases (31;32). Diffuse diabetic glomerulo-

sclerosis is less specific for diabetic glomerulosclerosis than nodular glomerulosclerosis. The nodular lesions of diabetic glomerulosclerosis are also known as Kimmelstiel-Wilson lesions.

Glomerular hyalinosis is common in diabetic glomerulosclerosis. These hyaline lesions may result from insudation or exudation of plasma proteins from vessels followed by entrapment in the matrix. The hyalinosis can occur anywhere in the glomerular tufts, but there are two characteristic patterns: capsular drops and hyaline caps (also known as fibrin caps). Capsular drops are spherical accumulations of hyaline material adjacent to or within the Bowman's capsule. The hyaline caps occupy the capillary lumen instead of being attached to Bowman's capsule.

Arteriolosclerosis and arteriosclerosis often accompany diabetic glomerulosclerosis. Arteriolar hyalinosis at the glomerular hilum is present in diabetic glomerulosclerosis and typically affects both the afferent and efferent arterioles. Hypertensive hyaline arteriolar sclerosis affects the afferent but not the efferent arteriole (33).

The earliest tubular change is thickening of tubular basement membrane that is analogous to the glomerular basement membrane thickening. When the disease progresses, tubules become atrophic and, fibrosis and chronic inflammation are present in the interstitium. Mononuclear cells can also be found in the interstitium in diabetic nephropathy. Inflammatory infiltrates of the interstitium, predominantly by T lymphocytes and macrophages, have been described (34). These chronic tubulointerstitial changes are not specific to diabetic nephropathy.

Pathological classification of diabetic nephropathy

Although for many kidney diseases pathological classification schemes exist (35-37), for diabetic nephropathy no uniform internationally accepted classification scheme has been developed yet. Classification schemes improve communication between and among renal pathologists and clinical nephrologists, provide logistical structure for prognostic and interventional studies and assist clinical management and efficiency (38).

In the past, several attempts have been made in classifying diabetic nephropathy. In 1959, Gellman *et al.* (39) proposed a systematic evaluation examining glomeruli, tubules, arterioles and the interstitium of kidney biopsies with diabetic nephropathy. The unique feature of this paper was that it presented for the first time associations between histopathological findings in renal biopsies with diabetic nephropathy and

severity of clinical parameters (39). Due to its extensive and elaborate nature, this evaluation system turned out to be unsuitable for practical usage.

More recently, in 1993, Gambara *et al.* (40) suggested to distinguish three classes of type 2 diabetes related nephropathy: class 1, typical diabetic glomerulopathy; class 2, aspecific glomerular and tubulointerstitial lesions, and class 3, different glomerular diseases superimposed on diabetic lesions. This system does not distinguish in severity of the lesions; it also contains a rather broad 'aspecific' category.

In 1996, Fioretto *et al.* (41) proposed another categorization of lesions for diabetic nephropathy in type 2 diabetes, also distinguishing between three classes: Class I, normal or near normal renal structure; Class II, typical diabetic nephropathy; Class III, atypical diabetic nephropathy. Although this system proved useful in evaluating renal biopsies for research purposes, it is not practical for clinical use. There are four drawbacks to this system:

- it does not discriminate between damage from other causes (e.g. vascular damage) and diabetic nephropathy,
- it does not distinguish in severity of the lesions,
- it is only suitable for 'proven' diabetic nephropathy at the electron microscopy level,
- the category 'atypical' is too broad to make a reliable evaluation of the biopsy.

In 2002, Mazzucci *et al.* (42) published an evaluation system for type 2 diabetic nephropathy that was based upon Gambara's scheme from 1993 (40). Mazzucci *et al.* (42) revised the Gambara scheme by re-naming class 2 into a category mainly characterized by changes related to vascular damage. In addition to this revision, they split class 3 into two subtypes: 3a) glomerular diseases superimposed on diabetic glomerulosclerosis and 3b) glomerular disease as the only renal change. They concluded that a renal biopsy should play a central role in management of type 2 diabetic patients with proteinuria.

None of these classification schemes were used in clinical practice. A standardized classification could encourage international uniformity in classifying diabetic nephropathy, facilitate experiments, be applied in multi-center clinical trials, and ultimately lead to improvement in the care of diabetic nephropathy. A proposal for such a classification scheme, based on severity of diabetic nephropathy, is made in chapter 2 by a group of experts.

Part II – Genetics in diabetic nephropathy

Francis Harry Compton Crick, James Dewey Watson and Maurice Hugh Frederick Wilkins received the Nobel Prize in Medicine for their discoveries in the molecular structure of DNA in 1962. Since then, many techniques have been developed to investigate variations in this DNA molecule in relation to disease. Several distinctions in genetic variation between individuals can be made based on variation in the DNA sequence and allele frequency. At the DNA level, there are insertion/deletions, copy number variations, microsatellites, and single nucleotide polymorphisms (SNP). Insertion/deletion are insertions or deletion in a certain part of the DNA sequence. Copy number variation is the same but larger, also referred to as duplications and insertions. Microsatellites are repeated sequences of which the CA repeat is the most common one. A single nucleotide polymorphism is a variant in a single nucleotide. Furthermore, based on allele frequency, the distinction is made between a polymorphism (minor allele frequency greater or similar to 1%) and a mutation, which is more rare (minor allele frequency <1%).

In the beginning, genetic mapping was used to search for causal mutations of diseases that run in families and are inherited by the principles of Mendel. These Mendelian diseases, also called monogenetic disorders, are caused by a mutation in a single gene. Duchenne muscular dystrophy is an example of a Mendelian disease. The disorder is caused by a mutation in the gene *DMD*, which encodes for the protein dystrophin, an important structural component within muscle tissue. Due to a single gene mutation the protein is not produced adequately, leading to the severely disabling phenotype of Duchenne. In contrast to Mendelian diseases, complex diseases are disorders in which the cause is considered to be a combination of several genetic effects and environmental influences. For example, type 1 and 2 diabetes, diabetic nephropathy and cardiovascular disease are such complex diseases.

Genetic mapping

Genetic mapping in the last century has resulted in a rapid increase in the understanding of disease pathology in many Mendelian diseases. These were found by a family-based approach based on the principle of linkage. Linkage is the tendency of certain loci or alleles to be inherited together. In family based linkage studies, it has been investigated which DNA markers (microsatellites) are more often inherited in affected family members compared to unaffected family members. When a genetic marker is

tightly linked (i.e. often inherited) with the disease, additional DNA markers near this marker are sought and studied. This process is often referred to as positional cloning and finally leads to the gene involved in the disease. This is a most useful approach in Mendelian disease, but in complex disease one gene does not ultimately lead to the disease. Therefore some relatives might be affected without the risk allele of the genetic variant and unaffected relatives might have this risk allele. Especially when a risk allele and disease are common (> 5% allele frequency), the inheritance pattern of the risk allele might not be so informative. An impractically large number of families would be needed to study a complex disease to find a small enough region to ultimately lead to one gene.

A candidate gene is a gene which is suspected to be involved in diabetic nephropathy based on the literature. To investigate a candidate gene association studies are used. Association studies investigate whether a risk allele occurs more frequently in subjects with the disease than in individuals without the disease. In contrast to familial studies, in association studies a problem called population stratification can occur. Population stratification refers to the problem that results in genetic association studies are due to ancestral differences in cases versus controls instead of related to the disease. Population stratification can only occur when either allele frequency or the disease risk differs between ancestral backgrounds. Another problem is that a clear biological explanation beforehand is needed to choose such a candidate gene and from Mendelian diseases we have learned that most genes found, were completely unexpected.

A new approach, developed to investigate complex disease, is the genome wide association scan (GWAS). This is an association study of the genome with common SNPs used as genetic markers. Since many SNPs are present in the human genome, this gives a good coverage of the human genome. A great advantage of the GWAS is that it does not need a biological explanation beforehand. It maps the genome with common SNPs either directly involved in disease pathology or indirectly through tagSNPs, which are potentially linked with risk alleles. Another advantage is that population stratification can be corrected for in this approach, as with the many SNPs the ancestry of the individual can be determined.

Models in complex genetics

Three models have been proposed to explain the genetic component in complex disease; the common disease-common variant model (CD-CV model), the rare alleles of major

effect (RAME) model and the infinitesimal model.

The CD-CV model assumes that common variants contribute to risk, each explaining a small proportion of disease liability. Due to their high frequencies, these variants may explain a large part of the population risk. The genome-wide association scan (GWAS), based on the CD-CV model, has proven to be powerful (43). An example of a common disease variant consistently replicated is *APOE* in Alzheimer's disease and heart disease (44). In contrast, the RAME model postulates that common diseases are in fact genetically heterogeneous and caused by *de novo* mutations. A rare variant in each individual (or family) with large effect may contribute to risk. Support for this model comes from studies of rare genetic variants in the high density lipoprotein C (HDL-C) gene (45;46). Rare variants were more often present in patients with low HDL-C (<fifth percentile) than with high HDL-C (>95th percentile). These findings were replicated in an independent study population (45). That the GWAS provides solid evidence for novel gene associations does not ultimately mean that the CD-CV model explains the genetic risk in complex diseases. Common SNPs identified by GWAS could be associated with rare mutations. Therefore, some common SNPs may be a proxy for a rare variant with large effect. Rare variants with large effect may provide clear information on disease etiology.

The infinitesimal model assumes that liability for disease follows an asymptotic distribution, as shown for the continuous trait height. Hundreds of genes would be involved, covering a wide range of frequencies (47). This model has gained support in GWAS, where odds ratios of 1.2 may each account for a small fraction of the liability.

Genetic susceptibility of diabetic nephropathy

In type 1 diabetes it has clearly been shown that some patients develop diabetic nephropathy within the first fifteen years and after that the incidence decreases (4). This suggests that some patients seem to be more susceptible to develop diabetic nephropathy. This is less clearly shown in type 2 diabetes as probably many die of cardiovascular disease before reaching the age to develop diabetic nephropathy. Several studies indicate that a separate genetic risk factor exists for diabetic nephropathy in type 1 or type 2 diabetes. Evidence for a genetic predisposition is that diabetic nephropathy seems to aggregate in certain families (48;49). Furthermore the prevalence of diabetic nephropathy varies significantly among ethnic groups (48).

Genetics in diabetic nephropathy

The first studies performed in the search for genes in diabetic nephropathy were linkage analyses in family studies. Several genome-wide linkage scans have been published in diabetic nephropathy in type 1 and type 2 diabetes. Although most of these analyses evaluated small numbers of families from different ethnic groups, several consistent regions of linkage have been detected (50-56). These regions can be helpful in choosing candidate genes, but these linkage studies were underpowered for directing to one specific gene.

In genetic association studies several candidate genes for diabetic nephropathy have been investigated. The frequently investigated genes were the genes involved in the renin-angiotensin-system. The most studied variant, the insertion/deletion polymorphism in intron 16 of the gene coding for angiotensin-converting enzyme (ACE), has shown to have a small effect in a meta-analysis of 53 studies (57). Furthermore, genetic variants in genes coding for aldose reductase (58), endothelial nitric oxide (59-61), manganese superoxide dismutase (62), vascular endothelial growth factor (63), TGF-β (64), apolipoprotein E (65-67), inflammatory cytokines (68) among several other candidates were studied in relation to diabetic nephropathy. A broad definition of diabetic nephropathy was used in these studies, from hyperfiltration and microalbuminuria to biopsy proven diabetic nephropathy.

Combining the two approaches, also referred to as large scale mapping, has led to some success. A good example is the *CNDP1* gene. A region on chromosome 18q22-22.3 was found to be associated with diabetic nephropathy in large Turkish families who were not treated for this disease, making this an ideal population to study for this purpose (50). Genes of this relatively small region were searched for up- or downregulation in diabetic nephropathy. A few genes were differentially expressed in diabetic nephropathy and one of these genes was the *CNDP1* gene. Genetic variations were sought in this gene and tested in a genetic association study. A polymorphism in exon 2 was found to be associated with diabetic nephropathy (26). This is an example of a successful finding, however, most found regions in diabetic nephropathy are larger. Furthermore, microarray analyses are known for the high false-positivity rate and finding the right variant is also a challenge considering multiple testing. Therefore, only few successful examples of this approach are known.

A few GWAS have been performed in diabetic nephropathy (69;70), leading to

several variants in or near genes which were not hypothesized before. Examples are genetic variants in the engulfment and cell motility 1 gene (*ELMO1*) (71) and cysteinyl-tRNA synthetase gene (*CARS*) (70). Most of the found variants will not prove to be causal and the relevance of these findings still needs to be assessed.

Part III - *CNDP1*, carnosinase and carnosine in diabetic nephropathy

Carnosine

In 1900 carnosine (β-alanine-L-histidine), as the name implies, was first isolated from meat by Gulewitsch and Amiradzibi (72). Related compounds to carnosine are anserine, homocarnosine, ophidine, carcinine and N-acetyl carnosine. In the human body, carnosine and related compounds are found in high concentrations in muscle and nervous tissue, but are also present in several organs such as the kidney, liver and small intestine. In the muscle, carnosine makes up a significant fraction of the water soluble nitrogen-containing compounds. The most convincing role of carnosine in the muscle was considered to be the control of intracellular hydrogen ion concentration. It was presumed that this property explains its predominant association with white, fast glycolytic type IIb muscles which possess relatively few mitochondria. Therefore these fibers will more often need anaerobic activity, which generates lactic acid. The importance of carnosine as a physicochemical buffer within human muscle was examined by calculating its buffering ability over the physiological pH range. From the range of carnosine concentrations observed (7.2-30.7 mmol/kg dry muscle mass), it was estimated that the dipeptide could buffer between 2.4 and 10.1 mmol H^+/kg dry mass over the physiological pH range 7.1-6.5, contributing on average, approximately 7% to the total muscle buffering. This suggests that in humans, in contrast to many other species, carnosine is of only limited importance in preventing the reduction in pH observed during exercise (73). Further evidence that carnosine is more than a physiological buffer, comes from Severin and co-workers (74;75). They showed that after addition of carnosine in medium surrounding a fatigued skeletal muscle, the muscle working was restored and because of special precautions in their study, it was shown that it did not depend on the pH-buffering capacity of carnosine.

The protective effect of carnosine can also be explained by its antioxidant properties.

Carnosine is capable of preventing the accumulation of oxidized products derived from the lipid components of biological membranes (73;76;77). This is rather surprising considering that carnosine is water soluble and remote from the site at which the peroxidation of membrane lipids takes place. However, both carnosine and anserine potentiate the effect of lipid soluble antioxidants such as vitamin E (78). Detailed studies of the time course of lipid peroxidation in the sarcoplasmic reticulum suggest that the ability of carnosine to inhibit the accumulation of thiobarbituric acid-reactive products is mediated by the result of direct interaction of the dipeptide not only with free radicals generated within the system, but also with primary molecular products of the lipid peroxidation (76). The antioxidant effect of carnosine has been demonstrated at both cell and tissue level as well as in suspensions of lipids derived from cell membranes. Carnosine is able to suppress peroxidation induced both enzymatically and non-enzymatically and eliminates the products of free radical reactions. These effects are not confined to the muscle and are accomplished by membrane stabilizing action as shown by preservation and recovery of intact cell membrane structures.

Kohen *et al.* (79) have demonstrated that these compounds can act as antioxidants as a result of their ability to scavenge single oxygen, peroxyl radicals and hydroxyl radicals. Carnosine and its analogues have been shown to be efficient chelating agents for copper and other transition metals. Since human skeletal muscle contains one-third of the total copper in the body (20-47 mmol/kg) and the concentration of carnosine is relatively high, the complex of carnosine:copper was thought to be of biological importance. Kohen *et al.* results indicated the complex of copper:carnosine can dismute superoxide radicals released by neutrophils (79). Furthermore, carnosine has shown to attenuate oxidative damage to DNA molecules (80) in the presence of iron and copper ions.

Carnosine has also been shown to have AGE inhibitory actions; protein crosslinks induced by methylglyoxal (a precursor of AGE) were found to be eliminated in the presence of carnosine (81;82). Although the mechanism by which carnosine inhibits the formation of AGE is unknown, it is likely that the free amino group derived from β-alanine competes with the amino groups of proteins in their reaction with precursors of AGEs (75). Apart from the abovementioned protective capacities, carnosine has also shown to inhibit formation of foam cells *in vitro* (83) and is an natural ACE inhibitor (84).

While carnosine is mainly found in skeletal muscle, homocarnosine concentration

is higher in the brain. It has been postulated that carnosine is a neurotransmitter. However, no receptors for carnosine have been found in nervous tissues (85). The role of carnosine in the brain is still under debate (86).

Investigating this dipeptide gained popularity for its anti-aging potential; it suppresses cultured human fibroblasts senescence and delays aging in senescence-accelerated mice (87). The exact reason why carnosine has this effect is not known, but it is probably due to the combination of abovementioned protective effects. In humans, so far no relation with carnosine and longevity have been found (88). Carnosine has also been investigated in age-related diseases such as Alzheimer (89) and Parkinson disease (90), but also in a wide variety of disorders and diseases, such as autism (91), cataract (92) and, cancer (93). The exact role of carnosine in these diseases still needs to be established.

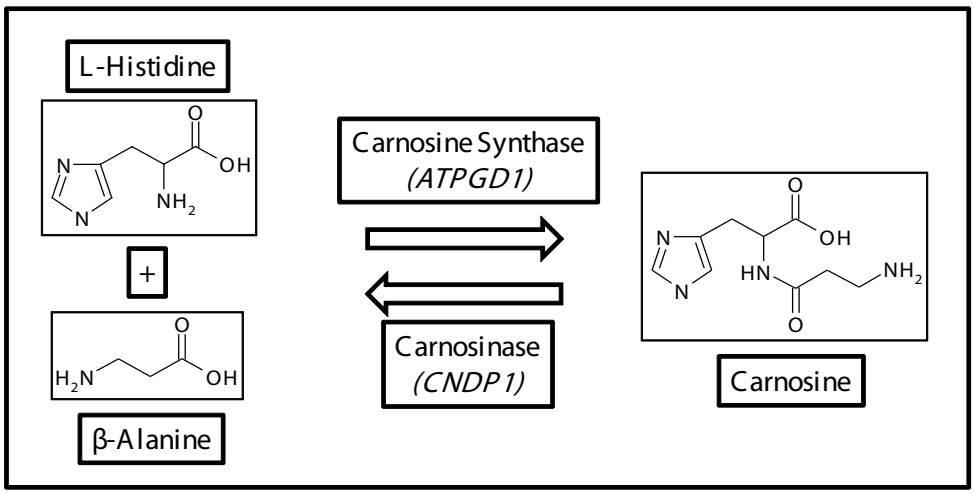

Figure 2. Carnosine metabolism

Carnosine metabolism

Carnosine and related compounds are produced by carnosine synthase of which the genetic sequence has recently been identified (94) as ATP-grasp domain containing protein 1, *ATPGD1*. Carnosine is synthesized by muscle cells, glial cells and oligodendrocytes (85). The kidney brush border also possesses a carnosine transport system (95;96). The majority of circulating carnosine is internalized by proximal tubular epithelial cells of the kidney via oligopeptide transporters Pept1 and Pept2 (97;98). Dibutyryl cyclic AMP and other agents that can, directly or indirectly, activate cyclic AMP-dependent protein

kinases strongly lower the rate of carnosine synthesis (99).

Carnosinase degrades carnosine into L-histidine and β-alanine (figure 2). Classical carnosinase was first described by Hanson and Smith (100), who found hog kidney carnosinase in 1949. Later, a distinction was made in two isoforms of carnosinase; serum carnosinase (also known as carnosinase-1) and tissue carnosinase (also known as carnosinase 2) (101). Teufel *et al.* showed that in fact there were two different genes coding for each of the enzymes (102). Tissue carnosinase is not capable of degrading carnosine under physiological condition in humans and has been shown to have rather a broad specificity (102). Therefore, this enzyme should be renamed into 'non-specific dipeptidase' and serum carnosinase into the genuine carnosinase (94;102). The following part will only describe data on genuine carnosinase. The physiological function of this carnosinase seems to be the hydrolysis of homocarnosine in the brain and the splitting of carnosine and anserine in the blood stream. Carnosinase is present in plasma and the brain and is specific to carnosine and related compounds.

Carnosinase activity is nearly absent in neonates (103), low in children (although protein level are similar in children and adults) and seems to increase with age (104). Women have slightly higher carnosinase levels than men (105). In the literature, some cases have been described with serum carnosinase deficiency, leading to severe neurological symptoms such as epilepsy, myoclonic seizures, microcephaly, blindness and mental retardation (103;106-108). On the other hand, subjects with carnosinase deficiency without any symptoms (103) have been described and the degree of serum carnosinase deficiency does not seem to correlate with the severity of the disease (106). However, it seems evident that even without a clear dose-response relationship, carnosinase deficiency is often associated with mental retardation (106).

Carnosinase-carnosine metabolism in diabetes and diabetic nephropathy

The most important clue that carnosine metabolism plays a role in diabetic nephropathy comes from genetic studies (26;50). A region on chromosome 18q22-22.3 was found to be associated with diabetic nephropathy in large Turkish families (50). A polymorphism in the *CNDP1* gene, which encodes serum carnosinase and is located in the chromosomal region 18q22-22.3, was found to be associated with diabetic nephropathy (26). Freedman *et al*. (25) confirmed this finding in European Americans with end stage diabetic nephropathy due to type 2 diabetes, but the association between diabetic nephropathy and the *CNDP1* gene could not be confirmed in patients with diabetic

nephropathy due to type 1 diabetes (27;28). The polymorphism is a microsatellite of CTG repeats (coding for the amino acid leucine) in exon 2 of the *CNDP1* gene, which varies between 0, 4, 5, 6, 7 and 8 repeats. Alleles with null, four and eight repeats are rare (25;26;88). Patients with homozygosity for 5 leucine repeats demonstrated a reduced susceptibility (2.0-fold reduced risk) for developing diabetic nephropathy due to type 2 diabetes compared with individuals with 6–8 repeats (25;26). Increasing numbers of leucine repeats in the leader peptide, have shown to increase the secretion of serum carnosinase (109) and to lead to higher serum carnosinase activity (26). Carnosines and related dipeptides are generally known for their protective effects. In the pathogenesis of diabetic nephropathy oxidative stress plays a central role. Therefore, of particular interest in relation to diabetic nephropathy is the scavenging of reactive oxygen species effect of carnosine (110). Formation of AGE is increased in a diabetic environment and has a major role in the development of diabetic nephropathy (111). Carnosine is able to scavenge precursors of AGE, such as methylglyoxal, and facilitates the degradation of AGEs (81). Furthermore, carnosine has shown to inhibit angiotensin converting enzyme (84), potentially lowering blood pressure. Elevated blood pressure could accelerate the development of diabetic nephropathy. Finally, TGF-β induced synthesis of extracellular matrix components (26) is thought to induce fibrosis and can be reduced by carnosine. This might explain why lower carnosinase levels (because of the 5-5 *CNDP1* homozygous genotype) leading to high circulating carnosine concentrations, are protective in diabetic nephropathy (figure 3).

Carnosine is mainly present in meat and fish and therefore it is interesting to see that vegetarians have higher levels of a precursor of AGE, which carnosine can scavenge, than carnivores. Furthermore, carnosine content increases with exercise (112;113), which has been shown to be body beneficial in diabetes, apart from the fact that it decreases mass content (114).

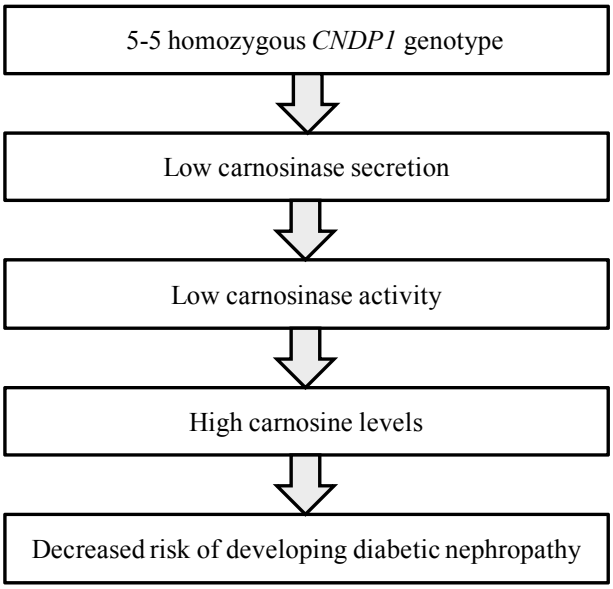

Figure 3. Hypothesis of *CNDP1* genotype in relation to diabetic nephropathy

Carnosine in animal models

With exception of the Goldhamster, carnosinase is not present in serum of rodents. However, rodents have a functional carnosinase coded by *CNDP1*, but it is not excreted from the cell into the serum. Several studies have been performed with carnosine treatment in rodents with diabetic nephropathy (115-117). Ob/ob mice, a leptin-deficient mouse used as a model for type 2 diabetes and diabetic nephropathy, were made transgenic for human carnosinase under a liver-specific promoter (117). Human carnosinase was present in serum of these transgenic mice and in this unique model, carnosinase could be overexpressed. When the animals were treated with carnosine, the hyperglycemia was reduced. The histological features of the kidney, serum creatinine levels and albumin creatinine levels in the carnosine-treated mice versus the untreated mice did not differ. These data suggest that the protective effect of the 5-5 homozygous genotype in diabetic nephropathy is primarily regulated by improved glycemic control (117). The obese Zucker rat, used as a model for metabolic syndrome (which includes type 2 diabetes) associated with kidney damage, was also treated with carnosine. Carnosine led to a reduced albumin excretion and improved kidney function in these rats. Furthermore, carnosine reduced the plasma triglycerides and insulin resistance but not plasma glucose (115). The characteristic pattern of lipid abnormalities in

patients with diabetes consists of moderate elevation in triglycerides, low high-density cholesterol values, and an increase in small dense low-density lipoprotein particles. Possibly in this rat model the improvement of the kidney is primarily due to improvement in the lipid abnormalities. In male streptozocin-treated Sprague-Dawley rats, used as a model for type 1 diabetes with diabetic nephropathy, the treatment with carnosine was also investigated. In these rats carnosine treatment reduced the amount of albumin excretion, but not plasma glucose (116). This is in contrast to streptozocin-treated Balb/cA mice, which did show improved glycemic control, lower triglyceride concentrations after carnosine treatment. In the latter study, the effect of carnosine on the kidney was not investigated (118). Clearly, more studies have to be done in mice to establish the role of carnosine in diabetic nephropathy.

Outline of this thesis

This thesis, with as a central theme diabetic nephropathy, comprises 7 chapters. In **chapter 2** a histological classification of diabetic nephropathy is proposed, classifying diabetic nephropathy according to severity of the disease. The idea is that this will induce clinical and pathological uniformity, leading to better patient care and scientific communication. As diabetic nephropathy has a strong genetic component, **chapter 3** covers the genetic variants reproducibly associated with diabetic nephropathy in a meta-analysis study. Chapters 4, 5 and 6 focus on one genetic variant known to be involved in diabetic nephropathy, the leucine repeat in exon 2 of the *CNDP1* gene. In **chapter 4**, a population of South Asians Surinamese immigrants, who have an increased risk of developing diabetic nephropathy, was investigated for the frequency of the protective 5-5 homozygous genotype of the *CNDP1* gene. **Chapter 5** describes the relation between the *CNDP1* genetic variant and gender in diabetic nephropathy in three independent diabetic nephropathy groups. In **chapter 6** the relation between the *CNDP1* genotype with glomerular kidney diseases other than diabetic nephropathy was investigated. **Chapter 7** relates more to the physiological aspect of carnosine, investigating determinants such as gender, age, diet, *CNDP1* genotype, carnosinase and testosterone in relation to carnosine content in muscle.

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2

Pathologic Classification of Diabetic Nephropathy

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Abstract

Although pathologic classifications exist for several renal diseases, including IgA nephropathy, focal segmental glomerulosclerosis, and lupus nephritis, a uniform classification for diabetic nephropathy is lacking. Our aim, commissioned by the Research Committee of the Renal Pathology Society, was to develop a consensus classification combining type 1 and type 2 diabetic nephropathies. Such a classification should discriminate lesions by various degrees of severity that would be easy to use internationally in clinical practice. We divide diabetic nephropathy into four hierarchical glomerular lesions with a separate evaluation for degrees of interstitial and vascular involvement. Biopsies diagnosed as diabetic nephropathy are classified as follows: Class I, glomerular basement membrane thickening: isolated glomerular basement membrane thickening and only mild, nonspecific changes by light microscopy that do not meet the criteria of classes II through IV. Class II, mesangial expansion, mild (IIa) or severe (IIb): glomeruli classified as mild or severe mesangial expansion but without nodular sclerosis (Kimmelstiel–Wilson lesions) or global glomerulosclerosis in more than 50% of glomeruli. Class III, nodular sclerosis (Kimmelstiel–Wilson lesions): at least one glomerulus with nodular increase in mesangial matrix (Kimmelstiel–Wilson) without changes described in class IV. Class IV, advanced diabetic glomerulosclerosis: more than 50% global glomerulosclerosis with other clinical or pathologic evidence that sclerosis is attributable to diabetic nephropathy. A good interobserver reproducibility for the four classes of DN was shown (intraclass correlation coefficient = 0.84) in a test of this classification.

Introduction

Diabetic nephropathy (DN) is a major cause of ESRD, and the incidence of diabetes mellitus is rising rapidly.[1] Pathologic classifications exist for several kidney diseases such as lupus nephritis,[2] focal segmental glomerulosclerosis,[3] and IgA nephropathy,[4] yet there is no uniform classification for DN. Classification schemes lead to better communication between renal pathologists and clinicians, provide logistical structure for prognostic and interventional studies, and improve clinical management and efficiency.[5]

In 1959, Gellman *et al.*[6] first reported an overview and clinical correlation of findings on renal biopsies from patients with DN. Before their study, the renal pathology in patients with diabetes mellitus was only described at autopsy. Gellman proposed an elaborate systematic evaluation examining glomeruli, tubules, arterioles, and the interstitium that was unsuitable for practical use. More recently, attempts were made to categorize patterns seen in DN after type 2 diabetes.[7–10] Gambara *et al.*[7] and Fioretto *et al.*[8] made basic distinctions between typical and atypical DN as well as other glomerular diseases superimposed on DN.[7,8]

Although such schemes are useful for research biopsies, they also are not practical for clinical use. We decided to classify DN due to type 1 and type 2 diabetes together because there is substantial overlap with respect to histologic lesions and renal complications.[11–13]

Our aim was to develop a uniform classification system containing specific categories that discriminate lesions with various prognostic severities that would be easy to use. This proposal was launched by the Research Committee of the Renal Pathology Society in 2006 in San Diego and further discussed in Leiden in September 2008.

Presented here is a consensus classification of DN developed by a group of international experts.

CLASSIFICATION OF DN

It is essential to evaluate renal tissue using appropriate standards for renal biopsy. These include hematoxylin and eosin, periodic acid–Schiff (PAS), Masson trichrome, and periodic acid methenamine silver stains for light microscopy. Biopsies should contain at least 10 glomeruli, 14 excluding incomplete glomeruli along the biopsy edge. Immunofluorescence requires the use of antibodies against IgA, IgG, IgM, C3, C1q, and kappa and lambda light chains to rule out other renal diseases. Electron microscopy (EM) must be performed; specific guidelines are discussed below. All of these methods

are necessary for an accurate diagnosis of DN. DN should never be diagnosed without supportive clinical information, and a patient should carry a clinical diagnosis of diabetes mellitus to apply the classification. Furthermore, virtually any glomerular disease can accompany DN, postinfectious GN [9,15] and membranous glomerulopathy being the most common [11]; thus, any coexisting disorders should also be described. Four classes of glomerular lesions in DN are presented in Table 1.

Table 1: Classification system for diabetic nephropathy into 4 classes

Class	Description	Inclusion Criteria
I	Mild or non-specific LM changes and EM proven GBM thickening	Biopsy does not meet any of the criteria mentioned below for class II,III or IV
		GBM > 395 nm in female, and > 430 nm in male individuals of 9 years and older*
II a	Mild Mesangial Expansion	Biopsy does not meet criteria for class III and IV
		Mild mesangial expansion in more than 25% of the observed mesangium
II b	Severe Mesangial Expansion	Biopsy does not meet criteria for class III and IV
		Severe mesangial expansion in more than 25% of the observed mesangium
III	Nodular slerosis (Kimmelstiel-Wilson lesions)	Biopsy does not meet criteria for class IV
		At least one convincing Kimmelstiel-Wilson lesion
IV	Advanced diabetic glomeruloslerosis	Global glomerular sclerosis in >50% of glomeruli
		Lesions from classes I-III

LM = light microscopy, EM = electron microscopy, GBM = glomerular basement membrane, DN= diabetic nephropathy

* based on direct measurement of GBM width by EM, these individual cut-off levels may be considered indicative when other GBM measurements are used

Classes of Glomerular Lesions

Class I: Glomerular Basement Membrane Thickening.

If the biopsy specimen shows no or only mild, nonspecific changes by light microscopy that do not meet the criteria of classes II through IV [in effect, in the absence of mesangial expansion, nodular increases in mesangial matrix (Kimmelstiel–Wilson lesions), and global glomerulosclerosis of more than 50% of glomeruli] the biopsy is assigned to class I (Table 1 and Figure 1), in which by direct measurements with EM the glomerular basement membrane (GBM) on average is thicker than 430 nm in males 9 years and older and thicker than 395 nm in females. These cutoff levels are based on a deviation from normal GBM thickness plus 2 standard deviations as recently determined by Haas.[16] For children younger than 9 years old, we refer to Table 1 in the

paper by Haas.[16] Upper limits for normal GBM thickness vary with the methods used to measure GBM width. For instance, using the orthogonal intercept method, upper limits of normal GBM thickness are 520 nm for adult men and 471 nm for women.[17] In individual laboratories, the upper limits for normal GBM thickness have usually been established, and if other methods than direct GBM measurement are used, it is advised to use these locally established cutoff points.

Light microscopic changes in the GBM and epithelial foot process effacement by EM have no influence on the classification. Class I incorporates cases that have been called "normal or near normal DN" by Fioretto *et al.*,[8] but in our system, a certain degree of chronic and other reactive changes (*e.g.*, changes of arterionephrosclerosis, ischemic type changes, or interstitial fibrosis) are accepted as part of this category. Diagnosing DN in cases without characteristic light microscopic glomerular lesions may be difficult, especially when a thicker GBM is also seen with aging or hypertension. The presence of arteriolar hyalinosis may be helpful in these cases, although it is not a prerequisite.

GBM thickening is a characteristic early change in type 1 and type 2 DN[13] and increases with duration of disease.[21] GBM thickening is a consequence of extracellular matrix accumulation, with increased deposition of normal extracellular matrix components such as collagen types IV and VI, laminin, and fibronectin.[22,23] Such accumulations result from increased production of these proteins, their decreased degradation, or a combination of the two. GBM thickening may already be present in type 1 diabetes patients who are normoalbuminuric.[20,21] GBM thickening has even been described as a "prediabetic" lesion: In patients with proteinuria and isolated GBM thickening but without overt diabetes, 20% were positive on a blood test for diabetes at the time of biopsy, whereas 44% were diagnosed with diabetes at 6 months, and 70% at 2 years after the biopsy was taken.[24] Long-term glucose control and urinary albumin excretion (UAE) correlate strongly with basement membrane thickness.[25] In 1979, Jensen *et al.*[26] were among the first to measure GBM thickness using the orthogonal intercept method. In brief, a grid with eight evenly spaced intersecting lines (four horizontal and four vertical) is placed over a photomicrograph, and GBM measurements are made at each point that a line on the grid intercepts an endothelial-GBM interface. Currently, some laboratories use computer-assisted measurements by which the mean width is calculated from approximately 50 measurements of the GBM at five different locations. The GBM width is then compared with GBM width from normal subjects, as determined previously by Steffes *et al.*[27] and recently updated by Haas.[16] Ideally, glutaraldehyde-fixed, plastic resin-embedded tissue should be used for EM, keeping in mind that other

methods, particularly the reprocessing of paraffin tissue for EM, may cause artifactual GBM thinning as recently reported by Nasr *et al.*[28] If computer-assisted measurements are not available, we recommend doing direct GBM measurements as recently modified by Haas.[16]

Class II: Mesangial Expansion, Mild (IIa) or Severe (IIb).

Class II encompasses those patients classified with mild or severe mesangial expansion but not meeting inclusion criteria for class III or IV (Table 1 and Figure 1) and is analogous to the previously used term "diffuse diabetic glomerulosclerosis."

Mesangial expansion is defined as an increase in extracellular material in the mesangium such that the width of the interspace exceeds two mesangial cell nuclei in at least two glomerular lobules. The difference between mild and severe mesangial expansion is based on whether the expanded mesangial area is smaller or larger than the mean area of a capillary lumen. If severe mesangial expansion is seen in more than 25% of the total mesangium observed throughout the biopsy, the biopsy is classified as IIb. If this is not the case, but at least mild mesangial expansion is seen in more than 25% of the total mesangium, the biopsy is classified as IIa.

Expansion of cellular and matrix components in the mesangium is a hallmark of type 1 and type 2 DN.[13,18] It can be detected in some patients within a few years after the onset of type 1 diabetes.[20] When the mesangium expands, it restricts and distorts glomerular capillaries and diminishes the capillary filtration surface. In our classification, we do not distinguish between mesangial hypercellularity, matrix expansion, or "mesangiosclerosis"— any expansion of the mesangium that conforms to our definitions given above and in Table 1 belongs to class II.

Various indices have been proposed to describe the amount of mesangial expansion in DN. Mauer *et al.*[18] define mesangial expansion by mesangial fractional volume or volume density (VV), defined as the fraction or percentage of the cross-sectional area of the glomerular tuft made up by mesangium, expressed in the formula: Vv(mes/glom).[18] Using this formula, many correlations have been made between mesangial expansion and clinical parameters of DN, particularly showing highly inverse correlations exist between Vv(mes/glom) and GFR.[18,29,30] There is also a relationship between Vv(mes/glom) and UAE[18,29] and blood pressure.[31] Another index to express mesangial expansion is the so-called "index of mesangial expansion" (IME) for DN.[18] The IME is determined by a semiquantitative estimate of the width of mesangial zones in each glomerulus[18]:

grade 0 is normal, 1 is twice normal thickness, 2 is three times normal thickness, and so forth; half grades can also be assigned. The mean of the grades for each glomerulus for IME can thus be determined from a single biopsy. The IME closely correlates with the Vv(mes/glom).[18] Still, this is a rather elaborate method.

In other classifications, mesangial expansion is defined in more practical ways, such as in the new classification for IgA nephropathy in which it is defined as an increase in the extracellular material in the mesangium such that the width of the interspace exceeds two mesangial cell nuclei in at least two glomerular lobules.[4] Because interobserver agreement was tested in this study and found to be satisfactory using this definition, we decided to use the same definition for mesangial expansion in our classification for DN.

Class III: Nodular Sclerosis (Kimmelstiel–Wilson lesions).
If at least one convincing Kimmelstiel–Wilson lesion is found and the biopsy specimen does not have more than 50% global glomerulosclerosis it is classified as class III (Table 1 and Figure 1). Kimmelstiel–Wilson lesions appear in type 1 and type 2 diabetes as focal, lobular, round to oval mesangial lesions with an acellular, hyaline/matrix core, rounded peripherally by sparse, crescent-shaped mesangial nuclei.[32]

Paul Kimmelstiel and Clifford Wilson, a German and an Englishman who met at Harvard, first described nodular lesions in glomeruli from eight maturity-onset diabetes patients in 1936.[33] According to Cameron,[34] they barely noted the association with diabetes, and it was Arthur Allen who clarified the association in 105 patients with diabetes in 1941.[35] Nodular sclerotic lesions may also occur in the absence of DN that are clinically related to hypertension, smoking, hypercholesterolemia, and extrarenal vascular disease.[36] It is claimed that in the initial stage of developing nodular sclerotic lesions in DN, two important processes take place: lytic changes in the mesangial area called mesangiolysis and detachment of endothelial cells from the GBM.[37] Exactly how these two processes relate remains uncertain. Paueksakon *et al.*[38] detected fragmented red blood cells in Kimmelstiel–Wilson lesions, which supports the theory that microvascular injury contributes to the pathogenesis of these lesions.

Dissociation of endothelial cells may disrupt the connections between the mesangial area and the GBM. This process precedes expansion of the Kimmelstiel–Wilson lesion.[37] These lesions consist of an accumulation of mesangial matrix with collagen fibrils, small lipid particles, and cellular debris.[39] A completely developed Kimmelstiel–Wilson lesion destroys the normal structure of glomerular tuft with a decrease in mesangial cells,

especially in the central area.[37] In 1992, a graphic method of analysis of the position of Kimmelstiel–Wilson lesions demonstrated the nodules were distributed in a horseshoe-shaped area corresponding to the peripheral or intralobular mesangium,[40] excluding the possibility of hyperfiltration as being their main cause of development.

The presence of at least one Kimmelstiel–Wilson lesion associates with longer duration of diabetes and less favorable clinical parameters.[10,41] In a study of 36 patients with type 2 diabetes, patients with Kimmelstiel–Wilson lesions had more severe overall retinopathy and higher serum creatinine concentrations than those with mesangial lesions alone.[10] In a study of 124 Chinese patients with type 2 diabetes, patients with at least one Kimmelstiel–Wilson lesion had relatively long duration of diabetes mellitus, a poor prognosis, and frequent evidence of diabetic retinopathy.[41] Kimmelstiel–Wilson lesions are often found in combination with mesangial expansion. The occurrence of Kimmelstiel– Wilson lesions is widely considered transitional from an early or moderately advanced stage to a progressively more advanced stage of disease.[41,42] Therefore, in our classification, the occurrence of Kimmelstiel–Wilson lesions implies a separate class.

Class IV: Advanced Diabetic Glomerulosclerosis.

Class IV implies advanced DN and designates those biopsies with more than 50% global glomerulosclerosis in which there is clinical or pathologic evidence that the sclerosis is attributable to DN (Table 1 and Figure 1). Glomerulosclerosis in DN is the end point of multifactorial mechanisms that lead to excessive accumulation of extracellular matrix proteins such as collagen types I, III, and IV and fibronectin in the mesangial space, which through stages of mesangial expansion and development of Kimmelstiel–Wilson lesions finally result in glomerulosclerosis.[43] The clustering of sclerotic lesions in columns perpendicular to the kidney surface suggests that vascular factors relating to the interlobular arteries also contribute.[44]

Designation of class IV lesions in our classification system is restricted to those cases in which there is evidence for DN. This evidence can come from other lesions in the biopsy as described for classes I through III. The occurrence of hyalinosis of the glomerular vascular pole or a capsular drop may also be taken as evidence for the presence of DN. Alternatively, if DN is the likely clinical diagnosis (*e.g.*, by the presence of retinopathy) a biopsy with extensive glomerulosclerosis can also be classified as class IV. Glomerulosclerosis without evidence of DN should be mentioned as such in the conclusion of the pathology report but should not be assigned class IV.

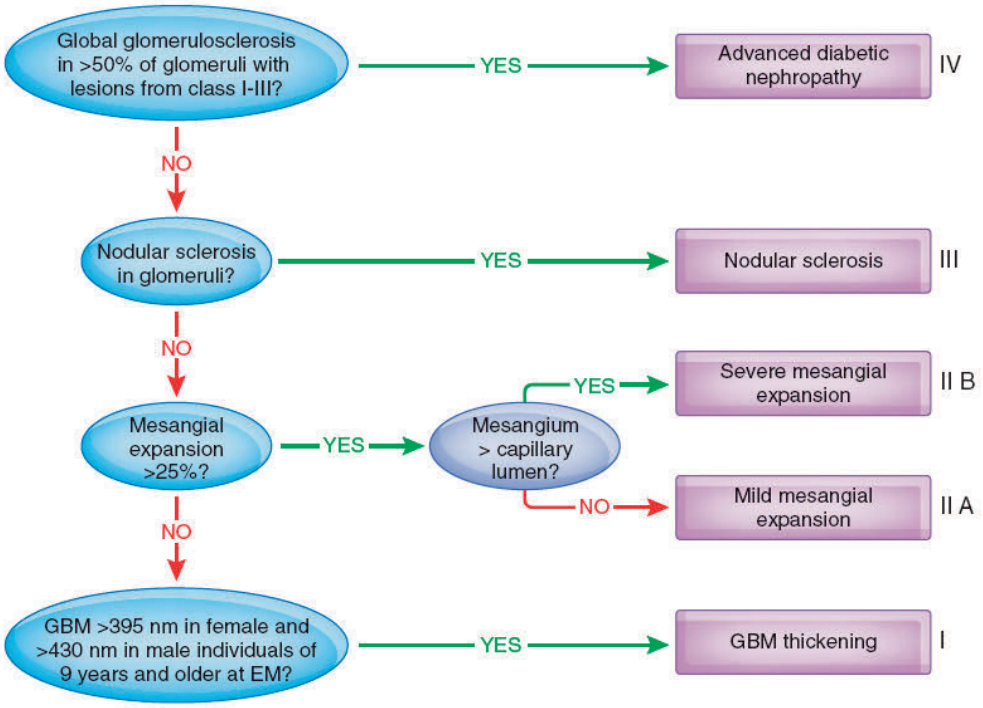

Figure 1. Flow chart for classifying DN.

Tubulointerstitial Lesions, Vascular Lesions, and Nondiabetic Glomerular Lesions

Tubular Lesions.

Concomitant tubular basement membrane thickening of nonatrophic tubules is apparent from the development of class II glomerular diabetic lesions and becomes more conspicuous in class III and IV, which is best seen in PAS or silver stains. Interstitial fibrosis and tubular atrophy (IFTA) follow glomerular changes in type 1 DN that ultimately lead to ESRD.[30] We score IFTA together as a percentage of the total involved area of interstitium and tubules (Table 2). A score of 0 is assigned when the biopsy specimen shows no IFTA, a score of 1 is assigned when less than 25% IFTA is present, a score of 2 is assigned when at least 25% but less than 50% of the biopsy has IFTA, and finally, a score of 3 is assigned when at least 50% IFTA is present, which is similar to the scoring in the recently published classification of IgA nephropathy.[4] Presence of mononuclear cells in the interstitium is a widely recognized finding in DN. Inflammatory interstitial infiltrates comprise T lymphocytes and macrophages.[45] In Table 2, we score 0 if interstitial infiltrates are absent, 1 if they only occur around atrophic tubules, and 2 if the inflammatory infiltrate is also in other areas than around atrophic tubules.

Table 2. Interstitial and vascular lesions of DN

Lesion	Criteria	Score
Interstitial lesions		
IFTA	No IFTA	0
	<25%	1
	25% to 50%	2
	>50%	3
interstitial inflammation	Absent	0
	Infiltration only in relation to IFTA	1
	Infiltration in areas without IFTA	2
Vascular lesions		
arteriolar hyalinosis	Absent	0
	At least one area of arteriolar hyalinosis	1
	More than one area of arteriolar hyalinosis	2
presence of large vessels	–	Yes/no
arteriosclerosis (score worst artery)	No intimal thickening	0
	Intimal thickening less than thickness of media	1
	Intimal thickening greater than thickness of media	2

Vascular Lesions.

According to Stout *et al.*,[46] hyalinosis of the efferent arteriole is relatively specific for DN, but hyalinosis of the afferent arteriole occurs in numerous other settings. Chronic cyclosporine nephropathy is a typical example in which arteriolar hyalinosis occurs outside DN.[47] Tracy *et al.* also report the presence of arteriolar hyalinosis in kidneys of young patients with coronary heart disease.[48] Efferent arteriolar hyalinosis is an important lesion by which DN is distinguished from hypertensive nephropathy.[49] However, most studies relate arteriolar hyalinosis to clinical parameters, not distinguishing between efferent and afferent arterioles, showing clear correlations with UAE and disease progression.[50,51] In Table 2 we score 0 if no arteriolar hyalinosis is present, 1 if one arteriole with hyalinosis is present, and 2 if more than one arteriole is observed in the entire biopsy. In addition to characteristic arteriolar hyalinosis, relatively nonspecific arteriosclerosis may be present in the biopsy specimen. Bohle and colleagues[45] found increases in vascular disease associate with more severe glomerular disease. Osterby *et al.*[52] use a so-called "matrix to media ratio" to investigate the role of arteriosclerosis and find this ratio is increased in patients with microalbuminuria, suggesting that arteriolar matrix accumulation occurs early in the course of DN. In Table 2 we score the most severely affected artery in the biopsy and assign a score of 0 if no intimal thickening is present, 1 if intimal thickening is less than the thickness of the media, and 2 if intimal

thickening is more than the thickness of the media. Isolated or significant medial thickness may be associated with concurrent hypertension.

Other Glomerular Lesions

In 1994, Stout *et al.*[46] defined "insudative lesions" as consisting of intramural accumulations of presumably imbibed plasma proteins and lipids within renal arterioles, glomerular capillaries, Bowman's capsule, or proximal convoluted tubules. Insudative lesions in Bowman's capsule are called capsular drop lesions, and in afferent and efferent arterioles they are called hyalinized afferent and efferent arterioles. In glomerular capillaries they are called fibrin cap lesions, although this term is considered obsolete and moreover is a misnomer because the lesion does not contain fibrin; we prefer the term hyalinosis for these lesions. Capsular drops are mainly located between the parietal epithelium and Bowman's capsule of the glomerulus.[11] Capsular drops are prevalent in advanced DN [53] and associate with disease progression.[54] The common belief, reviewed by Alsaad *et al.*,[49] is that capsular drops are specific but not entirely pathognomonic of DN. Stout *et al.*[46] report a prevalence of capsular drops in 5.3% of biopsies without diabetes. However, finding a capsular drop in a biopsy can help distinguish DN from other causes of glomerulosclerosis. By light microscopy, glomerular hyalinosis describes the same staining characteristics as the capsular drop lesion but it occupies the capillary lumen instead of being attached to Bowman's capsule. This lesion is not a specific finding in DN, because similar lesions are recognized in focal glomerulosclerosis, arterionephrosclerosis, and lupus nephritis.[55]

Finally, there is increasing recognition of abnormalities in the glomerulotubular junctions with focal adhesions called "tip lesions" and atrophic tubules with no observable glomerular opening (so-called "atubular glomeruli"). These lesions are typically found in more advanced stages of nephropathy associated with overt proteinuria.[30]

Interobserver Reproducibility

To assess the reproducibility of our consensus classification, a pilot study was performed in which five pathologists independently classified 25 renal biopsies with DN using PAS stains only into class I, II, III, or IV. Two pathologists scored all biopsies independently. Results of the raw data are given in Table 3. The reproducibility of the glomerular class score was evaluated using an intraclass correlation coefficient. Analyses were carried out using SPSS software (version 16, SPSS, Inc., Chicago, IL). There was disagreement in

seven cases: twice on a difference between class I and II, twice on a difference between class II and III, and three times on a difference between class IIa and IIb. Overall, the results seem satisfactory, resulting in an intraclass correlation coefficient of 0.84.

Flow Chart and Scoring Form

A flow chart was devised to help distinguish the four classes of DN (Figure 2). Supplemental Figure 1 shows the scoring form we recommend for classifying glomeruli in DN and for scoring of extraglomerular lesions or other features.

Table 3. Assessment of interobserver agreement for scoring of DN classes I to IV

Biopsy Nr	Observer 1	Observer 2	Observer 3	Observer 4	Observer 5	Agreement
1	IIA	I				N
2	III	III				Y
3	IIB	IIA				N
4	IIB	IIA				N
5	IIB	IIA				N
6	III	III				Y
7	IIB		IIB			Y
8	IIB		IIB			Y
9	IV		IV			Y
10	IIA		IIA			Y
11	IV		IV			Y
12	IIB		IIB			Y
13	III		III			Y
14				I	I	Y
15				IV	IV	Y
16				III	III	Y
17				III	III	Y
18				IIB	I	N
19	IIB				III	N
20	IIA				IIA	Y
21	III				III	Y
22	III				IIA	N
23	III				III	Y
24	III				III	Y
25	IIA				IIA	Y

Y = yes, N = no, intra-class correlation coefficient = 0.84

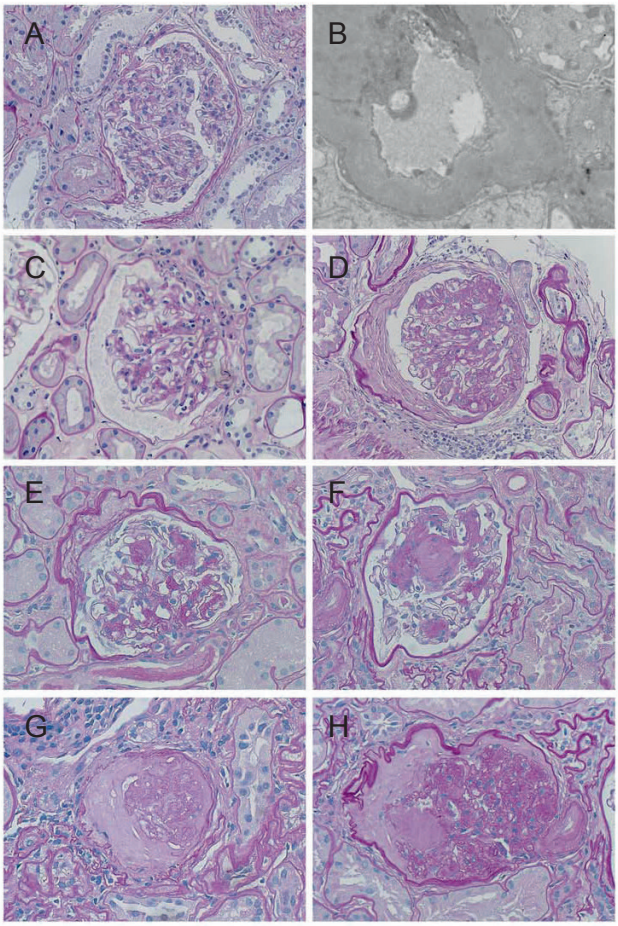

Figure 3. Typical examples of histomorphological lesions in DN.

A. glomerulus showing only mild ischaemic changes, with splitting of Bowman's capsule. No clear mesangial alteration. B. EM of this glomerulus: the mean width of the GBM was 671 nm (mean taken over 55 random measurements). EM provides the evidence for classifying the biopsy with only mild light microscopic changes into class I. C and D: glomeruli with mild and moderate mesangial expansion, respectively. In C, the mesangial expansion does not exceed the mean area of a capillary lumen, whereas in D, it does. E and F: in F Kimmelstiel-Wilson lesions. The lesion in E is not a convincing Kimmelstiel-Wilson lesion, therefore (on the basis of the findings in this glomerulus), findings are consistent with class IIb. For the purpose of the classification, at least one convincing Kimmelstiel-Wilson, like in F, needs to be present. G and H: global glomerulosclerosis. In H, signs of DN consist of hyalinosis of the glomerular vascular pole, and also a remnant of a Kimmelstiel-Wilson lesion on the opposite site of the pole. G is an example of glomerulosclerosis which does not reveal its cause (glomerulus from the same biopsy as H). For the purpose of the classification, signs of DN either histopathologically or clinically should be present in order to classify a biopsy with global glomerulosclerosis in > 50% of glomeruli as class IV.

Conclusions

We developed a classification scheme for DN consisting of four progressive classes supported by international consensus. The classification is based on glomerular lesions, with a separate evaluation for interstitial and vascular lesions. We chose a classification scheme based on glomerular lesions because these are relatively easy to recognize with good interobserver agreement as shown by our pilot data and because glomerular lesions best reflect the natural course of progressive DN.[49] Of course, glomerular and interstitial lesions contribute to the decline in renal function in DN and may be independent factors in the progression of DN[13]; however, many studies also show that severity of chronic interstitial and glomerular lesions closely associate.[12,18,19,56]

Although in some clinical practices there is a policy to only perform a renal biopsy to exclude causes of renal disease characterized by proteinuria other than DN, there is increasing demand to classify the severity of disease in those patients with pure DN. Our classification system for histopathologic lesions in DN can be used for patients with type 1 and type 2 diabetes, because it is now generally recognized that substantial overlap exists between these two types with respect to histologic lesions and renal complications.[12,13] Various studies also report different proportions of nondiabetic nephropathies in patients with diabetes and proteinuria.[7,57] The classification system proposed here is only for DN, but it can also serve to classify DN when it is complicated by another superimposed disease. Little is known about the pathogenesis of lesions developing in DN, but it is suggested that if we could unravel the various pathways ultimately leading to glomerulosclerosis in DN, this could open up new possibilities for intervention to prevent or forestall nephropathy.[43] Most likely, intra- and extraglomerular cells are involved in the progressive accumulation of extracellular matrix proteins in DN. We suggest that progression evolves from GBM thickening to mesangial expansion, Kimmelstiel–Wilson lesions, and global glomerulosclerosis, respectively, which is reflected in the four classes of our classification system. Using our system to evaluate protocol biopsies of patients with DN may further unravel the complex pathways of DN.

An important question for every histologic classification system is whether it is predictive of clinical outcome. As in other proposals for classifying renal disease (*e.g.*, for lupus nephritis[2] and focal segmental glomerulosclerosis[3]), we chose not to assess clinical outcome as part of this proposal. We feel validation should be done in separate prospective studies, preferably including protocol biopsies of patients with type 1 and type 2 diabetes and clearly defined clinical end points.

Disclosures

None.

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3

Genetic associations in diabetic nephropathy: a meta-analysis

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Abstract

Aims/hypothesis This meta-analysis assessed the pooled effect of each genetic variant reproducibly associated with diabetic nephropathy.

Methods PubMed, EMBASE and Web of Science were searched for articles assessing the association between genes and diabetic nephropathy. All genetic variants statistically associated with diabetic nephropathy in an initial study, then independently reproduced in at least one additional study, were selected. Subsequently, all studies assessing these variants were included. The association between these variants and diabetic nephropathy (defined as macroalbuminuria/proteinuria or end-stage renal disease [ESRD]) was calculated at the allele level and the main measure of effect was a pooled odds ratio. Pre-specified subgroup analyses were performed, stratifying for type 1/type 2 diabetes mellitus, proteinuria/ESRD and ethnic group.

Results The literature search yielded 3455 citations, of which 671 were genetic association studies investigating diabetic nephropathy. We identified 34 replicated genetic variants. Of these, 21 remained significantly associated with diabetic nephropathy in a random-effects meta-analysis. These variants were in or near the following genes: *ACE, AKR1B1* (two variants)*, APOC1, APOE, EPO, NOS3* (two variants)*, HSPG2, VEGFA, FRMD3* (two variants)*, CARS* (two variants)*, UNC13B, 'CPVL and CHN2'* and *GREM1*, plus four variants not near genes. The odds ratios of associated genetic variants ranged from 0.48 to 1.70. Additional variants were detected in subgroup analyses: *ELMO1* (Asians)*, CCR5* (Asians) and *CNDP1* (type 2 diabetes).

Conclusions/interpretation This meta-analysis found 24 genetic variants associated with diabetic nephropathy. The relative contribution and relevance of the identified genes in the pathogenesis of diabetic nephropathy should be the focus of future studies.

Introduction

Diabetes mellitus has rapidly increased to epidemic proportions over the past few decades. The number of patients with diabetes mellitus worldwide was estimated at 173 million in 2002 and is predicted to increase to 350 million cases by 2030 (1). Diabetes mellitus is associated with severe complications including nephropathy, neuropathy, retinopathy and accelerated cardiovascular disease.

Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) in developed countries (1). Although glycaemic control inversely relates to the degree of microvascular complications including diabetic nephropathy (2), some patients appear to be at increased risk. The majority of patients with type 1 diabetes mellitus will either develop diabetic nephropathy within the first 15 years after diagnosis or will remain relatively protected thereafter (3). Differential disease risk in diabetic nephropathy may be partly attributable to genetic susceptibility. Evidence for a genetic component to diabetic nephropathy comes from family studies displaying familial aggregation of diabetic nephropathy both in type 1 and in type 2 diabetes mellitus (4-6), as well as differences in the prevalence of diabetic nephropathy between ethnic groups (7,8).

The literature involving genetic associations in complex disease has been plagued by inconsistencies (9). Small sample sizes and false positive results were often responsible for lack of reproducibility (10). In addition, the prior probabilities of genetic associations are low. Therefore, the number of false positive associations generated by chance alone is high, particularly when low prior probabilities were not accounted for in the statistical analyses. (11). Incorrect phenotyping may also lead to spurious results. Thus independent replication of association remains essential in order to avoid false positive associations. The aim of this meta-analysis was to assess the pooled effect of genetic variants that have reproducibly been associated with diabetic nephropathy.

Methods

Eligibility criteria

We searched for studies comparing genetic variants in diabetes mellitus patients with diabetic nephropathy, relative to diabetes mellitus patients without diabetic nephropathy. We limited our analyses to studies investigating established and advanced diabetic

nephropathy. To be included, all cases in the report had to have diabetes mellitus with macroalbuminuria and/or overt proteinuria, ESRD attributed to diabetic nephropathy or biopsy-proven diabetic nephropathy. In addition, diabetic control participants had to have either: (1) normoalbuminuria; (2) normoalbuminuria or microalbuminuria after >15 years diabetes mellitus duration (microalbuminuria developing after >15 years diabetes mellitus duration is a poor predictor of diabetic nephropathy (3)); (3) stable kidney function (serum creatinine < 106.1 μmol/L) after >15 years of diabetes mellitus, irrespective of albuminuria.

Studies were excluded when the control group consisted of non-diabetic participants, since in that case genetic associations ascribed to diabetic nephropathy could have been due to diabetes mellitus. Follow-up and case–control studies were both eligible for inclusion in the meta-analysis.

Literature search and data collection

A search strategy was devised in collaboration with a trained librarian. The following databases were searched: PubMed (1949 to April 2010), EMBASE (OVID-version, 1980 to April 2010) and Web of Science (1945 to April 2010). The search strategy consisted of multiple queries combining: 'Diabetic Nephropathy' and 'Genes' or 'Polymorphisms'. For these two concepts, all relevant keyword variations were used. In addition, the names of specific genes and polymorphisms were combined with the topic 'Diabetic Nephropathy'. This search strategy was optimised for every database consulted, taking into account differences in the various controlled vocabularies and different database-specific technical variations. The search was performed in April 2010. To ensure maximum sensitivity, limits or filters were not placed on the searches. Language restrictions were not included in the initial search. References of other narrative and systematic reviews were also checked for relevant articles. The search strategy was updated if a reference was missing. The process was performed three times to ensure that no references were omitted.

Two authors (A. L. Mooyaart and E. J. J. Valk) of this study independently reviewed the titles and abstracts of the citations to identify genetic association studies. Genetic association studies were screened for whether the study contained a positive or a negative association between the genetic variant and diabetic nephropathy (association defined as significant at $p<0.05$). When a genetic variant was found to be associated with established or advanced diabetic nephropathy (either at the allelic or genotypic

level, including the recessive and dominant model) in two independent studies, that variant was considered to be a reproduced genetic variant. For these replicated variants, all other genetic studies were identified to estimate the effect of the variant on diabetic nephropathy, irrespective of *p* values.

Data extraction and analysis

The main outcome of the meta-analysis was the pooled odds ratio for the association between reproduced genetic variants and diabetic nephropathy. Odds ratios were calculated at allele level and not at genotype level. Of the reproduced genes, allele frequencies were extracted from studies. For single nucleotide polymorphisms (SNPs), the frequency of the minor allele was compared between diabetic nephropathy cases and non-nephropathy diabetic controls. For other genetic variants such as microsatellites, we compared the allele between cases and controls, as used in the literature and other meta-analyses (12, 13). The random-effects model was performed by default. Heterogeneity within the studies was estimated by the I^2, which is the percentage of the total variation across studies that is due to heterogeneity rather than chance. An I^2 of 25%, 50% and 75% was considered low, moderate and high respectively (14). Pre-specified stratified analyses were performed to explain heterogeneity or investigate whether the reported association was present in a subgroup. Stratified analysis was performed for diabetes mellitus type (type 1 or type 2), diabetic nephropathy stage (macroalbuminuria and/or overt proteinuria, established diabetic nephropathy and ESRD [advanced diabetic nephropathy]) (15) and ethnicity (European vs Asian origin). The subgroup analysis was only included in this study if the association between the genetic variant and diabetic nephropathy was reproduced in that subgroup. We tested for publication bias using the Begg and Egger test and provided funnel plots of all genetic variants which were reproducibly associated with diabetic nephropathy. It should, however, be noted that funnel plot asymmetry can have other causes than publication bias (16). Furthermore, the effect of ethnicity was assessed if there were sufficient data by metaregression. Most analyses were performed in Review Manager (RevMan) Version 5.0 (The Nordic Cochrane Centre, Copenhagen, Denmark; The Cochrane Collaboration, 2008), except for the analysis of publication bias and metaregression, which was performed in STATA 10.0 (StataCorp. 2007. *Stata Statistical Software: Release 10*. College Station, TX: StataC).

Results

Initial search and results

The initial literature search yielded 3455 citations, 671 of which were genetic association studies investigating diabetic nephropathy in humans (Fig. 1). In these studies, we identified 34 reproduced genetic variants in 24 genes associated with diabetic nephropathy. Data on at least one of these 34 variants were found in 132 articles, representing 153 studies. Only three follow-up studies were included. All other studies were case–control studies. The maximum number of studies in an article was five. References of all articles and details of these studies are shown in the Electronic supplementary material (ESM) Tables 1 and 2. The 132 articles were published between 1994 and 2010. The number of cases included in these articles ranged from four to 1572, and in a study from four to 656 cases. Of the 34 reproduced genetic variants, 21 genetic variants in or near 16 genes were significantly associated with diabetic nephropathy after random-effects meta-analysis (Fig. 2a). An overview of the pooled odds ratios of all reproduced variants in relation to diabetic nephropathy is shown in Fig. 2a, b. The odds ratios of the significant associations with diabetic nephropathy ranged between 0.48 and 0.78 for protective effects, and 1.12 to 1.70 for risk effects. Figure 3 contains an overview of the pooled odds ratios of all reproduced variants in relation to diabetic nephropathy among subgroups. Three reproduced variants were not significantly associated with diabetic nephropathy in the whole population after meta-analyses, but were associated in one subgroup: rs1799987 of *CCR5* and rs741301 of *ELMO1* in the Asian subgroup, and D18S880 of the *CNDP1* gene in patients with type 2 diabetes mellitus. Details of analyses of all assessed genetic variants are provided in Table 1. Forest plots of all individual genetic variants and funnel plots for publication bias, as well as results of meta-regression for ethnicity are shown in the ESM (ESM Figs 1–36). If the meta-analysis revealed a positive association between a given genetic variant and diabetic nephropathy, and more than ten studies investigating that variant in relation to diabetic nephropathy were available, a metaregression was performed. Only three genetic variants fitted the above-mentioned criteria (*ACE* rs179975, *AKRB1* CA repeat Z–2, *APOE* E2/3/4). In these variants, metaregression showed that ethnicity did not explain the heterogeneity (ESM Figs 1–36).

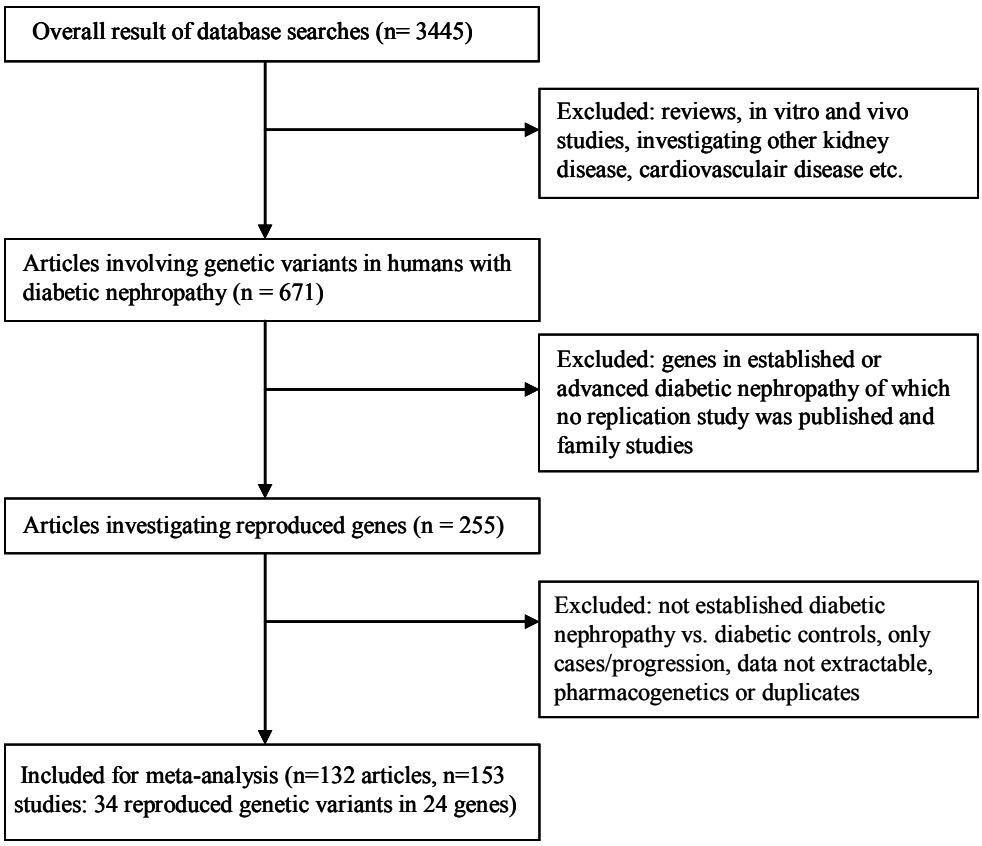

Figure 1. Flowchart showing how studies were selected for meta-analysis

Table 1. Details of reproduced variants after meta-analysis, including subgroups

Variants per gene	Minor allele	Total/subgroup	Studies (*n*)	Cases (*n*)	Controls (*n*)	OR (95%CI)	I^2 (%)	*p* value[a]
ACE								
rs179975	Deletion	Total	42	5721	7798	1.24 (1.12-1.37)	66	0.061
		Type 1 diabetes	14	2215	2685	1.13 (1.04-1.23)	44	-
		Type 2 diabetes	28	3506	5113	1.33 (1.16-1.52)	71	-
		ESRD	10	1405	1367	1.39 (1.21-1.6)	29	-
		Proteinuria	30	4071	5593	1.20 (1.07-1.36)	67	-
		Europeans	17	2660	3221	1.10 (0.99-1.22)	38	-
		Asians	19	2465	3397	1.28 (1.10-1.56)	65	-
ACACB								
rs2268388	T	Total	5	1007	900	0.83 (0.62-1.11)	77	0.624
ADIPOQ								
rs17300539	A	Total	3	1104	1138	1.37 (0.93-2.03)	67	0.117
AGT								
rs699	C	Total	21	4117	4800	1.10 (0.96-1.25)	71	0.85
		Type 2 diabetes	10	1966	2309	1.11 (0.85-1.45)	83	-
		Asian	8	1717	1933	1.10 (0.78-1.55)	87	-
AGTR1								
rs5186	C	Total	15	3220	3501	0.99 (0.91-1.08)	40	0.102
		European	10	1564	2038	1.06 (0.94-1.19)	36	-
		Type 1 diabetes	9	1525	1920	1.04 (0.92-1.17)	30	-
AKR1B1								
CA repeat	Z–2	Total	19	2237	3017	1.12 (1.02-1.24)	11	0.807
		Type 1 diabetes	10	1380	1308	1.12 (1.00-1.25)	16	-
		European	14	1654	1854	1.08 (0.95-1.21)	18	-
	Z+2	Total	17	1894	2005	0.83 (0.66-1.03)	59	0.805
		Type 1 diabetes	10	1380	1308	0.79 (0.68-0.92)	60	-
		European	11	1513	1557	0.81 (0.66-0.99)	40	-
rs759853	T	Total	9	1243	1933	1.40 (1.13-1.74)	67	0.144
		Type 1 diabetes	4	636	537	1.58 (1.01-2.46)	84	-
		European	6	854	913	1.45 (1.07-1.97)	75	-

APOC1								
rs4420638	G	Total	2	857	935	1.54 (1.29-1.83)	0	0.317
APOE								
E2, E3, E4	E2	Total	11	1257	1555	1.70 (1.12-2.58)	68	0.186
		Type 2 diabetes	5	368	751	2.21 (1.22-4.00)	42	-
		Asians	4	312	722	2.35 (1.29-4.30)	48	-
		Europeantype 1 diabetes	6	889	803	1.48 (0.84-2.58)	77	-
	E4	Total	11	1257	1555	0.78 (0.62-0.98)	40	0.186
CARS								
rs451041	A	Total	3	1052	2057	1.37 (1.21-1.54)	0	0.117
rs739401	C	Total	2	820	885	1.32 (1.15-1.51)	0	0.317
CCR5								
rs1799987	G	Total	9	2562	2965	0.81 (0.66-1.00)	85	0.012
		Asians	4	627	907	0.58 (0.43-0.76)	69	-
CNDP1								
D18S880	5L	Total	7	2603	3136	0.92 (0.82-1.01)	34	0.176
		Type 2 diabetes	2	344	329	0.77 (0.61-0.97)	0	-
'CPVL and CHN2'								
rs39059	G	Total	2	820	885	0.74 (0.64-0.85)	0	0.317
rs39075	A	Total	3	1052	2057	0.84 (0.62-1.14)	84	0.602
ELMO1								
rs741301	G	Total	3	1366	1219	1.31 (0.82-2.08)	91	0.602
		Asians	2	546	334	1.58 (1.28-1.94)	0	-
EPO								
rs1617640	T	Total	3	1618	954	0.67 (0.60-0.76)	0	0.117
		Type 1 diabetes	2	1244	715	0.67 (0.58-0.76)	0	-
FRMD3								
rs1888747	C	Total	3	1052	2057	0.74 (0.65-0.83)	0	0.602
rs10868025	A	Total	3	1052	2057	0.72 (0.64-0.81)	11	0.117
GLUT1								
rs841853	T	Total	7	867	1035	1.10 (0.89-1.35)	53	0.881
GREM1								
rs1129456	T	Total	2	859	940	1.53 (1.25-1.89)	0	0.317

Table 1. Continued

Variants per gene	Minor allele	Total/subgroup	Studies (*n*)	Cases (*n*)	Controls (*n*)	OR (95%CI)	I^2 (%)	*p* value[a]
HP								
Hp 1/2	Hp 1	Total	8	718	1056	0.81 (0.60-1.08)	62	0.322
HSPG2								
rs3767140	G	Total	4	732	381	0.72 (0.59-0.87)	0	0.384
		European type 1 diabetes	2	417	240	0.64 (0.49-0.84)	0	-
NOS3								
rs2070744	C	Total	2	273	450	1.39 (1.09-1.78)	0	0.216
rs3138808	a-deletion 393 bp	Total	8	1250	1368	1.31 (1.02-1.67)	53	0.317
		European type 1 diabetes	3	679	657	1.45 (0.97-2.17)	69	-
PPARG								
rs1801282	G	Total	7	2468	2394	0.84 (0.65-1.10)	78	0.024
PVT1								
rs11993333	T	Total	2	628	661	0.65 (0.39-1.10)	80	0.317
UNC13B								
rs13293564	T	Total	4	1572	1910	1.23 (1.11-1.35)	0	1.00
VEGFA								
rs833061	C	Total	2	242	301	0.48 (0.37-0.61)	0	0.317
No gene								
rs1041466	G	Total	3	1052	2057	1.38 (1.21-1.58)	0	0.602
rs1411766	A	Total	3	1052	2057	1.36 (1.20-1.54)	0	0.117
rs7989848	A	Total	3	1052	2057	1.32 (1.16-1.51)	0	0.117
rs9521445	A	Total	2	820	885	1.35 (1.18-1.55)	0	0.317
rs6492208	C	Total	3	1052	2057	0.85 (0.67-1.06)	0	0.117

[a]Begg test for funnel plot asymmetry, which is suggestive of publication bias

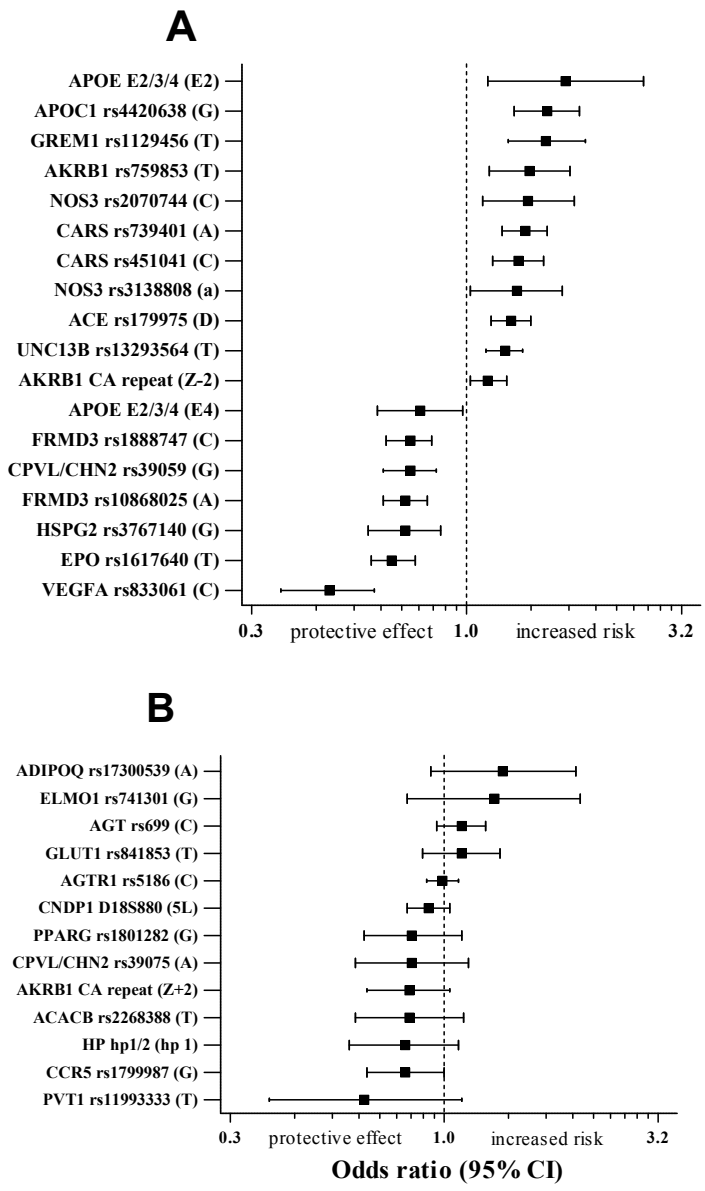

Figure 2. Genetic variants reproducibly associated with diabetic nephropathy. **a** All genetic variants in or near a gene that were reproduced in an independent study and significantly associated with diabetic nephropathy after meta-analysis. **b** All genetic variants in or near a gene that were reproduced in an independent study, but were not significantly associated with diabetic nephropathy after meta-analysis. Parentheses (*y*-axis labelling) contain the allele used in the comparison

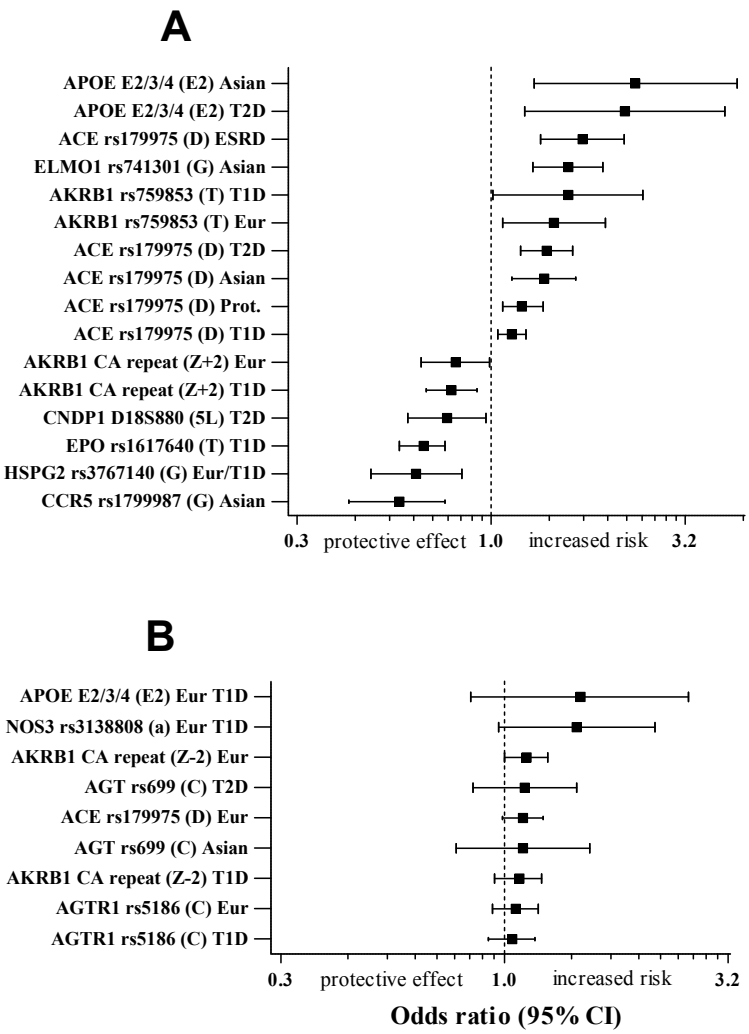

Figure 3. Genetic variants reproducibly associated with diabetic nephropathy in a subgroup. **a** All genetic variants in or near a gene that were reproduced in an independent study and significantly associated with diabetic nephropathy after meta-analysis in a subgroup. **b** All genetic variants in or near a gene that were reproduced in an independent study, but were not significantly associated with diabetic nephropathy after meta-analysis in a subgroup. Parentheses (*y*-axis labelling) contain the allele used in the comparison. The subgroup in which the genetic variant was reproducibly associated with diabetic nephropathy is shown in *y*-axis label as follows: Asian, T2D (type 2 diabetes), ESRD, T1D (type 1 diabetes), Eur (European), Prot. (proteinuria)

Genetic variants involved in the renin–angiotensin system

A variant in *ACE*, rs179975, was the most studied polymorphism in diabetic nephropathy, with 42 studies resulting in a pooled odds ratio of 1.24 (95% CI 1.12-1.37). The association between the deletion of the rs179975 polymorphism and diabetic nephropathy was reproduced in all subgroups. In the meta-analysis, the rs179975 polymorphism was associated with diabetic nephropathy in type 1 diabetes mellitus (OR 1.13 [95% CI 1.04-1.23]), type 2 diabetes mellitus (OR 1.33 [95% CI 1.16-1.52]), ESRD (OR 1.39 [95% CI 1.21-1.60]), proteinuria (OR 1.20 [95% CI 1.07-1.36]) and in the Asian subgroup (OR 1.28 [95% CI 1.10-1.49]), but not in Europeans. Other variants in the renin–angiotensin system that were also widely studied and reproduced, such as the rs699 variant of *AGT* with 21 studies and the rs5186 polymorphism of *AGTR1* with 15 studies, were not associated with diabetic nephropathy in the meta-analysis.

Genetic variants involved in the polyol pathway

The CA repeat and rs759853 in *AKR1B1* were studied in nineteen and nine studies, respectively. The CA repeat has a Z–2 allele thought to lead to an increased risk of diabetic nephropathy and a Z+2 allele thought to have a protective effect. The Z+2 allele and Z–2 allele were both reproducibly associated with diabetic nephropathy, but only the Z–2 allele remained associated in a combined meta-analysis with a pooled odds ratio of 1.12 (95% CI 1.02-1.24). Although reproducibly associated with diabetic nephropathy in 'type 1 diabetes mellitus' and 'European' subgroups, Z–2 was not associated with diabetic nephropathy in the meta-analysis in these subgroups. The Z+2 allele was associated with diabetic nephropathy in the 'type 1 diabetes mellitus' and 'European' subgroups (OR 0.79 [95% CI 0.68-0.92] and OR 0.81 [95% CI 0.66-0.99], respectively). The T allele in SNP rs759853 was associated with risk of diabetic nephropathy in the meta-analysis (OR 1.40 [95% CI 1.13-1.74]) and in the subgroups 'diabetic nephropathy due to type 1 diabetes mellitus' and 'Europeans' (OR 1.58 [95% CI 1.01-2.46] and OR 1.45 [95% CI 1.07-1.97], respectively).

Genetic variants involved in lipid metabolism

Two variants in genes each coding for two different apolipoproteins are reproducibly associated with diabetic nephropathy and remained associated with diabetic nephropathy in the meta-analysis: E2, E3, E4 polymorphism of *APOE* and rs4420638 near *APOC1*. The E2 allele is thought to lead to an increased risk of diabetic nephropathy

and the E4 allele is thought to have a protective effect. Both the E2 and the E4 allele were associated with diabetic nephropathy in the meta-analysis (OR 1.70 [95% CI 1.12-2.58] and OR 0.78 [95% CI 0.62-0.98] respectively). The E2 allele was also reproducibly associated with diabetic nephropathy in the subgroups 'type 2 diabetes mellitus', 'Asians' and 'European/type 1 diabetes mellitus' (all studies investigating Europeans had type 1 diabetes mellitus and vice versa), but only associated with diabetic nephropathy in the meta-analysis in the 'type 2 diabetes mellitus' and 'Asian' subgroups (OR 2.21 [95% CI 1.22-4.00] and OR 2.35 [95% CI 1.29-4.30], respectively). Rs4420638 near the *APOC1* gene was studied in two studies and was associated with diabetic nephropathy in the meta-analysis (OR 1.54 [95% CI 1.29-1.83]). Both studies contained type 1 diabetic nephropathy patients of European descent.

Genetic variants involved in inflammatory cytokines and angiogenesis

Rs1799987 of the *CCR5* (an inflammatory cytokine) gene was only associated with diabetic nephropathy in the Asian subgroup (OR 0.58 [95% CI 0.43-0.76]) consisting of four studies (n=1534), but not in the total group consisting of nine studies (n=5527). For the total group, funnel plot asymmetry was indicated by a significant Begg test.

Two genes involved in angiogenesis, *VEGFA* and *EPO*, each had a variant that was reproducibly associated with diabetic nephropathy. Rs833061 of *VEGFA* was associated with diabetic nephropathy in the meta-analysis in two studies (n=543) containing only type 1 diabetes mellitus patients of European origin (OR 0.48 [95% CI 0.37-0.61]). Rs1617640 of *EPO* was associated with diabetic nephropathy (OR 0.67 [95% CI 0.60-0.76]) in three studies (n=2773), also in the subgroup with type 1 diabetes mellitus patients (OR 0.67 [95% CI 0.58-0.76]).

Genetic variants involved in oxidative stress

Five genetic variants in four genes thought to be related to oxidative stress were reproducibly associated with diabetic nephropathy. The 1/2 polymorphism of *HP* and rs1801282 of *PPARG* were not associated with diabetic nephropathy in the meta-analysis. For *PPARG*, funnel plot asymmetry was observed (p=0.024) suggesting publication bias. The rs3138808 and the rs2070744 variants of *NOS3* were associated with diabetic nephropathy in the meta-analysis (OR 1.31 [95% CI 1.02-1.67] and OR 1.39 [95% CI 1.09-1.78] respectively). The 5L allele of *CNDP1* was associated with diabetic nephropathy only in the 'type 2 diabetes mellitus' subgroup (OR 0.77 [95% CI 0.61-0.97]).

Genetic variants in other pathways

Rs17300539 of *ADIPOQ*, which is believed to mitigate vascular damage, was not associated with diabetic nephropathy in the meta-analysis. Rs841853 of *GLUT1 (also known as SLC2A1)*, coding for a glucose transporter, did not show an association with diabetic nephropathy in eight studies (OR 1.10 [95% CI 0.89-1.35]). Rs1129456 of *GREM1*, which is involved in cell growth and differentiation, was associated with diabetic nephropathy (OR 1.53 [95% CI 1.25-1.89]) in two studies (n=1799). Rs3767140 of *HSPG2*, which is involved in maintenance of glomerular basement membrane electrostatic charge, was also associated with diabetic nephropathy in the meta-analysis (OR 0.72 [95% CI 0.59-0.87]), and additionally with diabetic nephropathy in Europeans with type 1 diabetes mellitus (OR 0.64 [95% CI 0.49-0.84]). Rs13293564 of *UNC13B*, thought to be involved in apoptosis, was associated with diabetic nephropathy in four studies (OR 1.23 [95% CI 1.11-1.35]).

Genetic variants identified by genome-wide association studies

Of the 14 genetic variants found to be reproducibly associated with diabetic nephropathy from genome-wide association studies (GWAS), ten remained associated in the meta-analysis. Rs2268388 of *ACACB*, rs11993333 of *PVT1*, rs39075 near '*CPVL* and *CHN2*', and rs6492208 (not near a gene) were not associated with diabetic nephropathy in the meta-analysis. Another variant near '*CPVL* and *CHN2*', rs39059, was associated with diabetic nephropathy in two studies (n=1705) (OR 0.74 [95% CI 0.64-0.85]). Rs741301 of *ELMO1* was associated with diabetic nephropathy in Asians with type 2 diabetic nephropathy (OR 1.58 [95% CI 1.28-1.94]), but not in combination with a third study of European type 1 diabetes mellitus patients. Rs451041 and rs739401 of *CARS* were associated with diabetic nephropathy in the meta-analysis (OR 1.37 [95% CI 1.21-1.54] and OR 1.32 [95% CI 1.15-1.51] respectively).

Rs1888747 and rs10868025 of *FRMD3* were associated with diabetic nephropathy (OR 0.74 [95% CI 0.65-0.83] and OR 0.72 [95% CI 0.64-0.81] respectively). Another four variants, rs1041466, rs1411766, rs7989848 and rs9521445, which do not lie near a known gene, were associated with diabetic nephropathy in the meta-analysis (OR 1.38 [95% CI 1.21-1.58], OR 1.36 [95% CI 1.20-1.54], OR 1.32 [95% CI 1.16-1.51] and OR 1.35 [95% CI 1.18-1.55] respectively). The variants in *CARS, FRMD3, 'CPVL and CHN2'* and the five variants not near genes were only investigated in European participants with type 1 diabetes mellitus.

Discussion

In this meta-analysis, 21 genetic variants were associated with advanced diabetic nephropathy and three additional variants were associated within specific subgroups. Meta-analysis of several individual genetic variants in relation to diabetic nephropathy has been performed previously, but this is the first complete overview assessing for all genetic variants that are reproducibly associated with the presence of diabetic nephropathy. This information could lead to improved insight into underlying pathogenetic mechanisms. Variants in or near *ACE, AKR1B1* (two variants)*, APOC1, APOE, EPO, NOS3* (two variants)*, HSPG2, VEGFA, FRMD3* (two variants)*, CARS* (two variants)*, 'CPVL and CHN2', UNC13B* and *GREM1*, as well as four variants not near known genes, were associated with diabetic nephropathy. *ELMO1*, *CCR5* and *CNDP1* were associated with diabetic nephropathy in a subgroup ('Asian's and 'type 2 diabetes mellitus' respectively). These results support a role for the following in the pathogenesis of diabetic nephropathy: renin–angiotensin system, polyol pathway, oxidative stress, inflammation, angiogenesis, glomerular filtration barrier defects, apoptosis, and cell growth and differentiation. Functional studies remain to be performed to establish the precise roles of these variants and pathways. Genetic variants initially identified using a genome-wide association approach in and near *FRMD3*, *CARS*, *ELMO1* and *'CPVL and CHN2'* were detected. The exact role of these genetic variants in relation to diabetic nephropathy requires further elucidation; many of these variants identified in GWAS will not prove to be causal.

Our analysis has some limitations. Publication bias is a concern in all meta-analyses. For this study, only published data in journals were used, discarding data published in congresses only. Negative studies are less likely to be published, potentially leading to an overestimation of effects. Moreover, non-significant genetic associations might have been underreported in published articles. Therefore, the effect estimates of the present study should be interpreted with caution, especially in cases where associations were based on small numbers of studies and/or small sample numbers. For example, the rs833061 variant in the *VEGFA* gene shows the strongest protective effect, but was investigated in two studies of moderate size. In these cases, additional studies are necessary to establish true effect sizes. It should also be acknowledged that by selecting only genetic variants that were associated with diabetic nephropathy and for which independent replication was available, genetic variants with smaller effect sizes may

have been missed, an effect that may have proven significant using pooled analyses. By selecting only those genetic variants reproducibly associated with diabetic nephropathy, we have tried to reduce the chances of describing false positive associations.

The studies included in the present analysis showed heterogeneity with respect to ethnicity, study design and phenotypes. For some of the analysis, the clinical heterogeneity was accompanied by statistical heterogeneity with an I^2 statistic of up to 91%. However, there is no fully accepted statistical measure that precisely determines clinical heterogeneity (16). To account for potential heterogeneity, random effects models were performed by default. These models assume that different studies have different true effects. To explore potential heterogeneity due to differences in ethnicity, a meta-regression was performed.

A study worth mentioning, which appeared after our inclusion date, is a paper by Maeda *et al.* (17), in which the authors investigated the variants found in the genome-wide association scan of the Genetics of Kidneys in Diabetes and Diabetes Control and Complications Trial studies (18) in four studies, of which three meet our criteria. We combined the data of Maeda *et al.* with results of the Genetics of Kidneys in Diabetes and Diabetes Control and Complications Trial studies. We found that only the rs451041 of *CARS*, and rs1041466, rs9521455 and rs1411766, which are not near a gene, were associated with diabetic nephropathy. In contrast to the Genetics of Kidneys in Diabetes and Diabetes Control and Complications Trial studies, which investigated Europeans with type 1 diabetes, Maeda *et al.* investigated diabetic nephropathy in type 2 diabetes in an Asian population. Therefore, the lack of association with diabetic nephropathy of the other variants could be explained by this difference.

The identification of diabetic nephropathy susceptibility variants can lead to novel biological insights and improved measures of individual aetiological processes, as indicated previously (19). Individual aetiological processes (personalised medicine) could allow preventive and therapeutic interventions in complex disease to be tailored to individuals on the basis of their genetic profiles. From prediction studies with genetic variants for type 2 diabetes mellitus, it has been shown that 20 established genetic variants in type 2 diabetes mellitus have an AUC of 0.54 (0.5 means no predictive value, 1.0 is perfect prediction), in contrast to the Framingham offspring and Cambridge risk scores (AUC of 0.78 and 0.72, respectively). Interestingly, addition of genetic information to phenotype-based risk models did not improve prediction (20). It is also possible that for diabetic nephropathy the genotypic risk does not exceed the risk contributed by

conventional risk factors (e.g. BMI, age, diabetes mellitus duration), which means that the predictive value of risk variants for diabetic nephropathy would be limited (21). Although genetic prediction and use of personalised medicine in diabetic nephropathy remains a new undertaking, prediction is likely to improve as additional disease variants are detected and replicated (22).

Novel biological insights may lead to development of new therapeutic targets, biomarkers and opportunities for disease prevention. Hypothesis-free approaches, such as GWAS, are most promising in this respect. At present, it seems wise to focus on assessing the relevance of previously detected genetic variants. As common SNPs associated with diabetic nephropathy and detected by GWAS may represent rare genetic variants with large effects, sequencing the regions surrounding highly significant and replicated genomic regions to detect rare variants appears to be reasonable. Follow-up *in vitro* and *in vivo* studies could then assess the functional relevance of these variants in diabetic nephropathy. In summary, our meta-analysis identified 24 genetic variants (in or near 16 different genes) associated with advanced diabetic nephropathy. These genetic variants are likely to represent true associations and further investigations to elucidate their functional relationship in diabetic nephropathy should be pursued.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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4

Lower frequency of the 5/5 homozygous *CNDP1* genotype in South Asian Surinamese

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Abstract

We investigated the frequency of the 5/5 homozygous *CNDP1* (carnosinase) genotype, which was found to be associated with a reduced risk of developing diabetic nephropathy, in three ethnic groups in the Netherlands. Particularly interesting were the South Asian Surinamese, who have a high prevalence of diabetic nephropathy. Furthermore, we investigated the association between this gene and carnosinase activity in South Asian Surinamese and whether carnosinase was expressed in the kidney.

We genotyped 290 South Asian Surinamese, 532 African Surinamese, and 472 white Dutch in a cross-sectional population study. Furthermore, an independent cohort of South Asian Surinamese was genotyped. In this population, carnosinase activity was measured in serum. Immunostaining and in-situ hybridization for *CNDP1* were performed on kidney tissue.

Both South Asian populations had lower frequencies of the 5/5 homozygous genotype than African Surinamese and white Dutch (23.0%, 27.2%, 38.2%, and 41.3%, respectively; chi-square, $p<0.001$). This genotype showed a lower carnosinase activity in South Asian Surinamese (Wilcoxon rank-sum, $p=0.03$). *CNDP1* was expressed in the kidney.

South Asian Surinamese have a lower frequency of the 5/5 homozygous genotype, which was associated with lower carnosinase activity. Our study provides an indication that South Asian Surinamese are genetically at risk for developing diabetic nephropathy.

Introduction

Diabetic nephropathy (DN) is the leading cause of end stage renal disease. Data from the UK prospective Diabetes Study group demonstrated that approximately 25% of patients with type 2 diabetes develop microalbuminuria or progressive DN within 10 years [1].

Several studies show a higher incidence of DN in certain families and races [2-4]. For example, the South Asian Surinamese, an ethnic minority who migrated from Suriname to the Netherlands but originally descended from India and Bangladesh, show an 8-times higher frequency of diabetes type 2 and a 3-fold higher risk of developing DN, a 40-fold higher risk of developing end-stage DN due to type 2 diabetes and a 1.5-fold faster disease progression than white Dutch [5,6]. The question arises whether these differences may in part be due to increased susceptibility because of a genetic predisposition.

As DN is a multi-genetic disease several susceptibility loci and candidate genes have been found (reviewed in [7]). One of the susceptibility loci for DN was identified by Vardarli *et al.* [4], who evaluated 18 Turkish families with type 2 diabetes and nephropathy in a family-based linkage study. A major susceptibility locus was found (LOD score 6.1) on chromosome 18q22.3-q23 for DN. This finding was confirmed by sib pair analysis on African Americans [8] and Pima Indians [4]. In a search for candidate genes within the quantitative trait locus (QTL) on chromosome 18q, Janssen *et al.* detected significant evidence for association between DN and *CNDP1*, which encodes for carnosine dipeptidase (also called serum carnosinase), in patients from Germany, the Netherlands, Qatar, and the Czech Republic [9]. Freedman *et al.* confirmed this finding in European Americans [10], but found a different polymorphism of the *CNDP1* gene in African Americans [11]. Interestingly, the association between DN and *CNDP1* could not be confirmed in patients with DN due to diabetes type 1 [12].Diabetes type 2 patients, who are homozygous for 5 copies of a trinucleotide repeat encoding for leucine in the leader peptide on exon 2, demonstrated a 2.56-fold reduced risk for DN compared to individuals with more leucine repeats (6-8 repeats) [9]. The presence of more than 5 leucine repeats has been shown to lead to higher serum carnosinase secretion [13] and higher carnosinase activity [9]. Serum carnosinase, a dipeptidase belonging to the M20-metalloprotease family, is the rate-limiting enzyme for hydrolysis of carnosine into beta-alanine and L-histidine. Carnosine is a reactive oxygen scavenger [14], exhibits

anti-AGE (advanced glycation end products) effects [15], a natural ACE (angiotensin converting enzyme) inhibitor [16] and reduces the synthesis of matrix components and TGF-β (transforming growth factor) in renal cell lines [9].

Our hypothesis is that the frequency of the leucine repeat in the *CNDP1* polymorphism differs with ethnic background and that South Asian Surinamese have an aberrant genotypic distribution of *CNDP1*, which could partly account for the reported higher risk for DN after the development of type 2 diabetes. Therefore, the primary objective was to investigate the frequency of the protective genotype, the 5/5 homozygous genotype, in South Asian Surinamese, African Surinamese (immigrants from Suriname who originally descended from Africa), and white Dutch in the Netherlands. The correlation between high carnosinase activity and more leucine repeats in the *CNDP1* gene has been found in whites, and the secondary objective of this study was to investigate whether the carnosinase activity was also correlated with the *CNDP1* genotype in South Asian Surinamese. Furthermore, to investigate the potential involvement in diabetic nephropathy we also determined whether carnosinase was expressed in the kidney.

Subjects, Materials and Methods

For this study, we analysed data from SUNSET (SUrinamese in the Netherlands: Study on Ethnicity and health) [17] and Hindinef (Hindustani Diabetic Nephropathy Study) [5]. Both studies have been approved by the institutional medical ethics committee in accordance with the Declaration of Helsinki.

SUNSET - SUNSET is a population-based, cross-sectional survey designed to obtain insight into the cardiovascular risk profile of three ethnic groups: South Asian Surinamese ('Hindustani', of South Asian origin), African Surinamese ('Creole' of African origin) and white Dutch [17,18]. Details of the recruitment, data collection and definitions of ethnicity are described elsewhere [19]. In brief, between 2001 and 2003 a random sample of non-institutionalised adults aged 35-60 years was selected from the Municipal Register of Amsterdam, the Netherlands. After informed consent, participants underwent an interview, followed by a physical examination, including donation of a fasting blood sample [19]. Ethnicity was subsequently determined based on self-identification.

Subjects who, did not participate in the interview and physical examination, who could not unequivocally be assigned to one of the three ethnic groups or who could not

be genotyped were excluded from the present study, leaving a total of 1294 persons for our present analysis. The 1294 that were genotyped consisted of 290 South Asian Surinamese, 532 African Surinamese and 472 white Dutch.

Hindinef - The Hindinef study is conducted in the Netherlands in the city of The Hague. The study was initially set up to investigate whether familial clustering of DN occurred in the South Asian Surinamese population. No evidence for familial aggregation of DN was found, and more South Asian Surinamese were collected but not yet described in this cohort.

The basic characteristics of the total cohort will be described in the results. The recruitment of the Hindinef study group were previously published [18].

Genotyping

DNA material from both SUNSET and Hindinef was genotyped as follows. A standard PCR protocol was used with primers 5-FAM-GCGGGGAGGGTGAGGAGAAC (forward) and GGTAACAGACCTTCTTGAGGAATTTGG (reverse). The denaturing, annealing and extension temperatures were 94°C, 60°C and 72°C, respectively. Fragment analysis was performed on the ABI3130 analyser (Perkin Elmer) to determine the fragment length corresponding with the different genotypes. Each peak corresponded with the number of leucine repeats on each allele. A 157 base pair product corresponded with 5 CTG codons encoding for 5 leucine repeats, and a product of 160 base pairs corresponds to 6 CTG codons encoding 6 leucine repeats. Homozygotes demonstrated a single peak, and homozygosity for the 5 leucine repeats in exon 2 is referred to as the protective genotype, 5/5 homozygous genotype. Samples with six or more leucine repeats were referred to as not having 5/5 homozygous genotype.

Determination of serum-carnosinase activity

Serum samples were obtained from a random group of 391 South Asian Surinamese of the Hindinef cohort. Serum-carnosinase activity was determined according to the method described by Teufel *et al.* [20]. Briefly, the reaction was initiated by addition of substrate (L-carnosine) to a serum sample and stopped after 10 minutes of incubation at 37°C by adding 1% sulphate salicylic acid. The maximum increase was used for determining the maximum activity. Liberated histidine was derivatized with *o*-phtaldialdehyde (OPA). Fluorescence was measured by excitation at 360 nm and emission at 460 nm.

The intra- and interassay variations were respectively 6% and 16%. The lowest carnosinase activity detectable was 0.117 µmol/ml/h.

CNDP1 expression in the kidney

Cadaveric donor kidneys were obtained from Eurotransplant. These kidneys were unsuitable for transplantation for technical or morphological reasons. For immunohistochemistry, paraffin slides were used. In brief, the slides were washed in PBS and incubated for 2 h at room temperature with the primary antibody (rabbit anti-*CNDP1*, generated as described by Teufel et al[20], diluted in 1% bovine serum albumin in PBS). After washing with PBS, the slides were incubated for 30 min with horseradish peroxidase–conjugated anti-rabbit Envision (DAKO, Glostrup, Denmark). The slides were again washed with PBS, and the staining was developed with diaminobenzidine. The RNA in situ hybridization was performed as previously described [21]. In brief, paraffin sections were pre-treated and hybridized with digoxigenin (DIG) labelled RNA probes for 16 hours. RNA hybrids were detected using mouse anti-DIG (Sigma-Aldrich Chemie, Steinham, Germany), rabbit anti-mouse Ig (1:50, Dako, Glostrup, Denmark), and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP, Dako).

Glomeruli were isolated as described earlier [22]. Whole kidney and glomerular RNA was isolated using Trizol®, converted to cDNA using AMV reverse transcriptase (Roche Applied Science). Real-time polymerase chain reaction [23] was used in combination with the following *CNDP1* primerset: forward: TTCAATCCGTCTAGTCCCTCACATG, reverse: TGCAATCCACGGGTGTAGTCC. Melting curve and sequence analysis were performed to confirm that we amplified the appropriate product.

Statistical Analysis

First, the characteristics of the SUNSET population and the frequency of 5/5 homozygous genotype and not having 5/5 homozygous genotype in both SUNSET and Hindinef were described per ethnic group. The difference in frequency of the 5/5 homozygous genotype between ethnic groups were assessed by means of the chi-squared test.

When the genotypes were divided in three groups with increasing number of leucine repeats, the statistical significance between the groups in carnosinase activity was determined by the Kruskal-Wallis test. The Wilcoxon sum-rank test was used to compare the carnosinase activity between the lowest number of leucine repeat group and the highest number of leucine repeat group and to compare the 5/5 homozygous genotype with the other genotypes.

A linear regression model was used to adjust for age and sex. The statistical analyses were all performed using SPSS 16.0 and Graphpad prism. A p-value < 0.05 was considered to be statistically significant.

Results

Characteristics of the SUNSET population and Hindinef population

The characteristics of the study populations are presented in Table 1. The white Dutch were slightly older than the South Asian Surinamese and African Surinamese and had the highest proportion of smokers. The African Surinamese had the highest proportion of female participants, as well as the highest median BMI (27.7 IQR 24.5-31.5) and a high frequency of high blood pressure (28.3%) compared to the other ethnic groups.

South Asian Surinamese had the highest frequency of diabetes (26.2%) and highest waist-hip ratio (0.96 IQR 0.90-1.00) compared to African Surinamese and white Dutch.

South Asian Surinamese of the Sunset and Hindinef study have a similar age and percentage male, BMI and waist-hip ratio, prevalence of diabetes and high blood pressure. Only in the Hindinef study group there were slightly less smokers.

Table 1. Characteristics of South Asian Surinamese, African Surinamese and white Dutch participants in SUNSET

	South Asian Surinamese		**African Surinamese**	**White Dutch**
	SUNSET	Hindinef		
	N=290	N = 456	N= 532	N= 472
Age (years)	44 (39-50)	42 (35-50)	43 (39-48)	48 (42-54)
Sex (male)	130 (44.8%)	196 (42.8%)	170 (32.0%)	233 (49.4%)
Smoking (current)	103 (35.9%)	103 (27%)	214 (40.9%)	212 (45.0%)
BMI (kg/m2)	26.6 (23.9-29.3)	26.2 (23.5-29.4)	27.7 (24.5-31.5)	25.5 (23.0-28.4)
Waist-hip ratio	0.96 (0.90-1.00)	0.94 (0.88-1.0)	0.90 (0.84-0.95)	0.90 (0.83-0.96)
High blood pressure (yes)	91 (31.5%)	133 (29.0%)	150 (28.3%)	83 (17.6%)
Diabetes Mellitus (yes)	76 (26.2%)	118 (25.8%)	68 (12.8%)	33 (7.0%)

N (%) or median (IQR)

Analysis of CNDP1 genotype frequency in South Asian Surinamese, African Surinamese and white Dutch

The *CNDP1* genotype distribution is demonstrated for each ethnic group in Table 2. The various genotypes were ranked according to the corresponding enzyme activities (Figure 2). The different genotypes are determined by fragment analysis. An example of a 5/5 homozygous genotype (A), a homozygous 6/6 genotype (B), a 5/6 heterozygous (C) and a 6/7 heterozygous (D) are seen in Figure 1. The frequency of 5/5 homozygous genotype in South Asian Surinamese (27.2%) was significantly lower than the frequency among African Surinamese (38.2%) and white Dutch (41.3%) in the SUNSET population ($p < 0.001$, Table 1). No differences were observed in the frequency between men and women (data not shown). The frequency of 5/5 homozygous genotype among the South Asian Surinamese with familial susceptibility for diabetes, the Hindinef population, was 23.0 % (105/456), which was similar to the frequency among the South Asian Surinamese in SUNSET, and significantly lower than the frequency among the African Surinamese and White Dutch in SUNSET (Table 2).

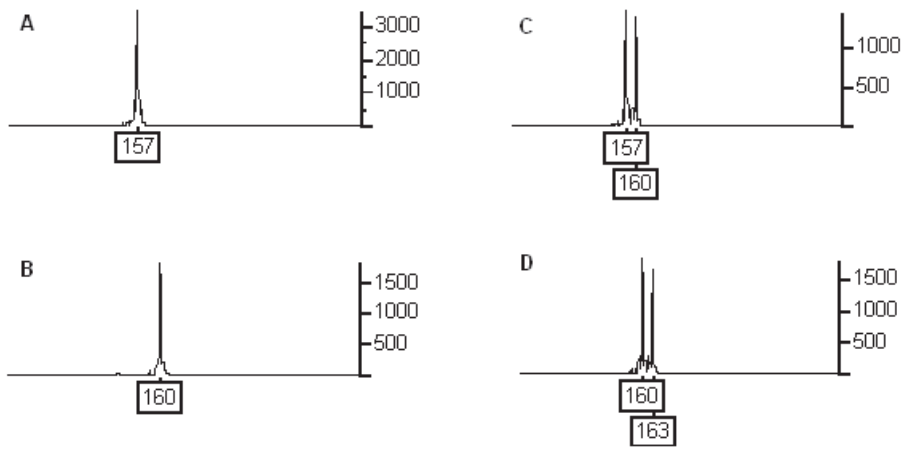

Figure 1. Measurement of the leucine repeat in exon 2 of the *CNDP1* gene

A = a fragment of 157 basepairs which corresponds to 5 leucine repeats in each allele (5/5 homozygous genotype), B = a fragment of 160 basepairs which corresponds to 6 leucine repeats in each allele, C = a fragment of 157 and 160 corresponding with 5 leucine repeats in one allele and 6 leucine repeats in the other, D = a fragment of 160 and 163 corresponding with 6 leucine repeats in one allele and 7 leucine repeats in the other.

Table 2. Distribution of *CNDP1* leucine repeats of South Asian Surinamese, African Surinamese and white Dutch participants

	South Asian Surinamese				**Creole Surinamese**		**White Dutch**	
	(SUNSET)		(Hindinef)					
	n=290	%	n = 456	%	n= 532	%	n= 472	%
4/4, 4/5, 4/6	3	1	0	0	18	3.4	2	0.4
5/5	79	27.2*	105	23.0*	203	38.2	195	41.3
5/6, 5/7	117	40.3	204	44.7	239	44.9	222	47
6/6, 6/7	91	31.4	147	32.2	71	13.3	51	10.8
7/7	0	0	0	0	1	0.2	2	0.4

* $p < 0.001$ chi-squared, comparing South Asian Surinamese with white Dutch and African Surinamese

Determination of serum carnosinase activity

The carnosinase activity was measured in 391 South Asian Surinamese of the Hindinef study. The genotypes were divided into three groups, the 5/5 homozygous genotype, the 5/6 and 5/7 heterozygous genotype, the 6/6 and 6/7 genotype. A clear correlation was found between carnosinase enzyme levels and the number of leucine repeats in the *CNDP1* gene (Figure 2). The carnosinase activity was significantly different between the three leucine repeat groups ($p = 0.03$). The 5/5 genotype differed significantly from the other genotypes ($p = 0.03$). The highest difference was found between the 6/6, 6/7 leucine repeat group and the 5/5 homozygous genotype ($p = 0.009$). The carnosinase activity did not seem to significantly differ with age and sex. When adjusted for age and sex in a linear regression model the association remained significant ($p = 0.04$). The apparent difference of significances is not due to age and sex variation but is a result of the different statistical tests.

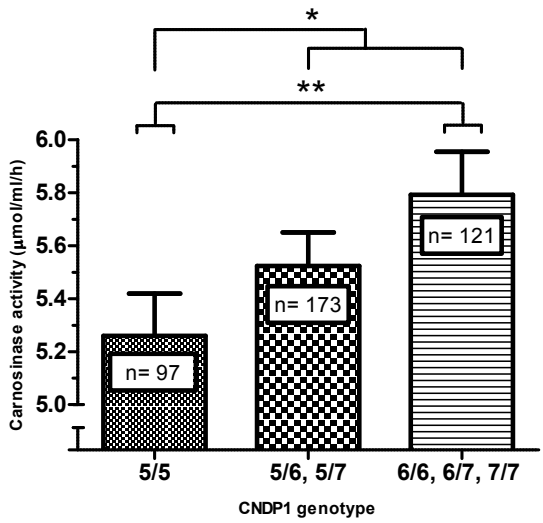

Figure 2. Mean serum-carnosinase activity in South Asian Surinamese categorised by amount of leucine repeats in both alleles.

Mean serum carnosinase activity with standard error of the mean divided in 3 genotype groups. '5-5' consists of 5-5 homozygous genotype, '5-6/5-7' consists of the genotype with 5 leucine repeats in one allele and 6 or 7 in the other allele, '6-6/6/7' consists of 6 leucine repeats in one allele and 6 or 7 in the other allele.

* < 0.05, ** < 0.01

CNDP1 expression in human kidney

Immunohistochemical staining and mRNA expression by in situ hybridization were performed on kidney tissue (Figure 3). Both protein and mRNA for *CNDP1* is expressed in glomerular cells and tubular epithelial cells. Real time PCR showed a specific expression of *CNDP1* in whole kidney, isolated glomeruli and mRNA.

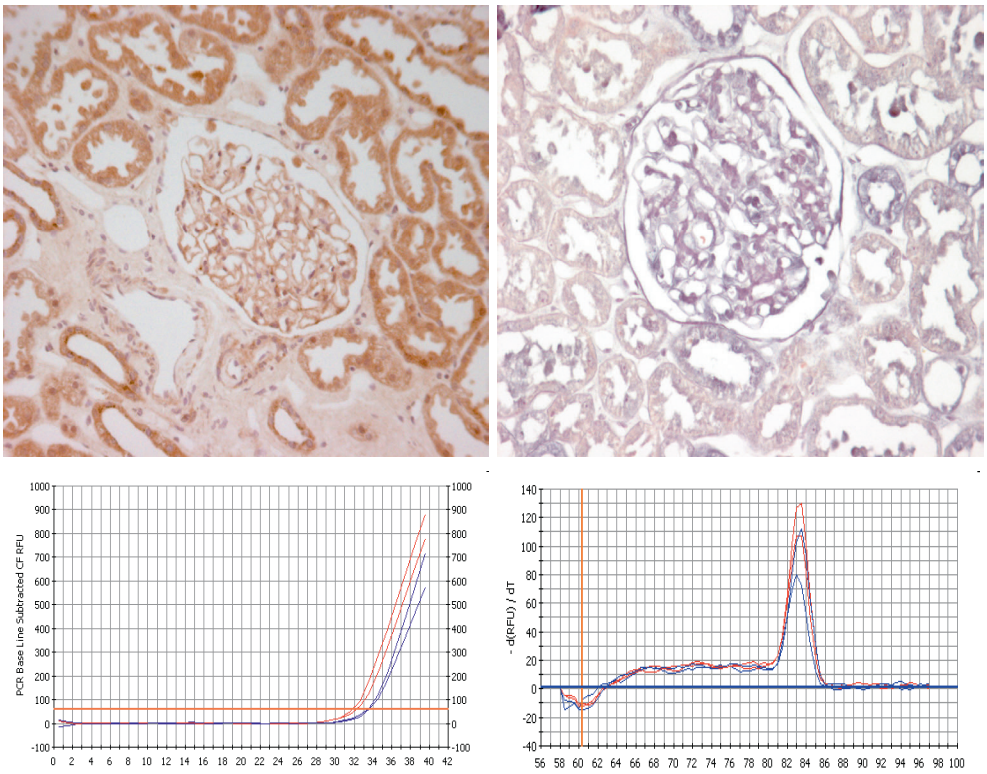

Figure 3. *CNDP1* expression in human kidney

Immunohistochemical staining (A) and mRNA expression by in situ hybridization (B) of *CNDP1* in normal kidney. Both protein and mRNA for *CNDP1* is expressed in glomerular cells and tubular epithelial cells. Real time PCR showed expression of *CNDP1* in both whole kidney (red lines) and isolated glomerular (blue lines) mRNA (C). Melting curve analysis showed specific amplification of *CNDP1* (D).

Discussion

This study reports the *CNDP1* polymorphism distribution in three ethnic groups in the Netherlands. Our study provides the first data on the frequency of 5/5 homozygous genotype among a population of South Asian origin. We found that the frequency of the protective genotype, the 5/5 homozygous genotype, was significantly lower in South Asian Surinamese compared to white Dutch and African Surinamese. The low frequency among South Asian Surinamese was confirmed in an independent South

Asian Surinamese cohort. Furthermore, we showed that, similar to Europeans, the carnosinase activity increases with the amount of leucine repeats among South Asian Surinamese and carnosinase was expressed in the kidney. This suggests a possible role in the development of DN in this population.

The white Dutch in our study show a frequency of the 5/5 homozygous genotype similar to that of healthy European control groups in previously published studies (41.3% in this study versus 36.8% in the study by Janssen *et al.* [9] and 38.6% in Freedman *et al.* [10].)

In African Americans, no association between reduced risk of DN and 5/5 homozygous genotype was found [10]. The frequency of 5/5 homozygous genotype among African Surinamese is similar to that of African Americans (38.2% and 35.9%, respectively). Other polymorphisms in the *CNDP1* gene have been found to play a role in the development of DN in African Americans [11]. These other polymorphisms in the *CNDP1* might play a role in African Surinamese as well, but were not included in this study.

The low frequency of the 5/5 homozygous genotype found in South Asian Surinamese observed in our study may be associated with the higher occurrence of DN in South Asian populations.

Janssen *et al.* found an association between the leucine repeat in the *CNDP1* gene and DN, and this finding was confirmed in a large study conducted by Freedman *et al.* in European American patients with DN and end-stage DN [9,10].

Functional studies provide evidence for the mechanism behind the association between 5/5 homozygous genotype and DN. The absence of 5/5 homozygous genotype has shown to lead to higher carnosinase levels and activity, thereby decreasing levels of carnosine [13]. Carnosine levels in blood are very low and vary during the day and therefore cannot be reliably ascertained (unpublished results). In our study we confirmed this increase in serum carnosinase activity with increasing number of leucine repeats in South Asian Surinamese. Carnosine has been shown to delay senescence in cultured human fibroblasts and temporarily reverse the senescence phenotype [24,25]. Moreover, several studies have demonstrated a likely anti-aging effect in vivo [26-28], which may also be related to the mechanism underlying vascular damage observed in DN. In that respect, in vitro supplementation of blood and plasma with carnosine has been shown to have an anti-oxidant effect is a promising finding [14]. If further developed, the implications of this discovery are potentially large, suggesting that

carnosine supplementation might be a possible therapeutic modality in the future for South Asian Surinamese subjects.

Before reaching a final conclusion, limitations deserve to be discussed. Selective recruitment or participation could have affected the reported frequency. The Hindinef population was originally selected for detecting familial clustering of DN. However, the frequency of 5/5 homozygous genotype was similar to the frequency in the South Asian population in SUNSET. We are therefore confident that our conclusion that the frequency of the 5/5 homozygous genotype is lower in South Asian Surinamese than in other ethnic groups living in the Netherlands is justified.

This is a population-based study and therefore provides no direct relation between diabetic nephropathy and the *CNDP1* gene in South Asian Surinamese but an indirect relation. Still, other investigators have found a clear relation between the *CNDP1* gene and diabetic nephropathy in Europeans [9] and European Americans [10]. South Asian Surinamese have found to have a significantly higher risk of diabetes type II and diabetic nephropathy [5,18]. The results of this study strongly suggest that the *CNDP1* polymorphism, given its distribution across ethnic groups, contribute to the higher risk of nephropathy in South Asian Surinamese.

Furthermore, DN is a multi-genetic disease and therefore it does seem unlikely that this gene is the only reason for the higher risk of developing DN of South Asian Surinamese. However, it might be one of the genes involved in the pathogenesis of DN in this ethnic minority in the Netherlands.

On the other hand, a powerful feature of this paper is that it presents data from a population-based survey among three ethnic groups in the same city. Selection due to clinic location and setting therefore did not occur. Another strong feature is that the findings from the SUNSET study could be confirmed in a second, independent study that was carried out in a different location (Hindinef). The similarity of the findings in both studies increase the power of the conclusions drawn concerning the frequency of the 5/5 homozygous genotype among the South Asian Surinamese.

In conclusion, South Asian Surinamese living in the Netherlands have a lower frequency of the protective genotype, 5/5 homozygous genotype, associated with a lower carnosinase activity. This polymorphism has proven to be protective for DN in Europeans and European-Americans and we showed that carnosinase was expressed in the kidney.

The specific role of 5/5 homozygous genotype in the occurrence of DN among South Asian origin populations remains to be further investigated, as carnosines or carnosine enhancers have the potential to become a therapeutic modality with great clinical relevance.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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5

Association between *CNDP1* genotype and diabetic nephropathy is sex-specific

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Abstract

Objective, The 5-5 homozygous *CNDP1* (carnosinase) genotype is associated with a reduced risk for diabetic nephropathy (DN). We investigated whether this association is sex-specific and independent of susceptibility for type 2 diabetes.

Research Design and Methods, Three separate groups of 114, 90, and 66 patients with type 2 diabetes and DN were included in this study, and compared with 93 patients with type 2 diabetes for >15 years without DN, and 472 population controls. The diabetes control group was used to determine an association in the three patient groups separately, and the population control to estimate the genotype risk (odds ratio [confidence interval]) for the population in a pooled analysis. The population controls were also compared with 562 patients with type 2 diabetes without DN to determine whether the association was independent of type 2 diabetes. The *CNDP1* genotype was determined by fragment analysis after PCR amplification.

Results, The frequency of the 5-5 homozygous genotype was 28%, 36%, 41% in the three DN patient groups, and 43% and 42% in the diabetic and population controls, respectively. The 5-5 homozygous genotype occurred significantly less frequently in women in all three patient groups compared with diabetic controls. The genotype risk for the population was estimated to be 0.5 [0.30–0.68] in women and 1.2 [0.77–1.69] in men. The 562 patients with type 2 diabetes without DN did not differ from the general population (P=0.23).

Conclusion, This study shows that the association between the *CNDP1* gene and diabetic nephropathy is sex-specific and independent of susceptibility for type 2 diabetes.

Introduction

Only 20– 40% of patients with type 1 or type 2 diabetes will develop diabetic nephropathy (DN), and if no signs of DN are present in the first 15 years after diagnosis of diabetes, the chance of ever developing DN is small (1). Furthermore, sibling studies show a strong familial component for development of DN (2;3), and certain ethnic groups seem to be at a greater risk of developing DN (2;4). These findings suggest that there is a genetic susceptibility component for DN.

Many genes are thought to be involved in DN (reviewed in (5)). One of the genes associated with DN in both patients with type 1 and type 2 diabetes is the *CNDP1* gene, which encodes serum carnosinase (6). This was confirmed in European Americans with end stage DN due to type 2 diabetes (7), but the association between DN and the *CNDP1* gene could not be confirmed in patients with DN due to type 1 diabetes (8;9) nor in African Americans (7).

Patients with type 2 diabetes of Caucasian origin with homozygosity for 5 leucine repeats in exon 2 demonstrated a reduced susceptibility for developing DN compared with individuals with 6–8 repeats (6;7;10). With increasing numbers of leucine repeats, the secretion of serum carnosinase has been shown to increase (11) and to lead to higher serum carnosinase activity (4;6). Serum carnosinase degrades carnosines and other histidine-containing dipeptides. Carnosines and related dipeptides are known for their reactive oxygen scavenging effects (12), to degrade advanced glycation end products (13), and reduce the TGF-β-induced synthesis of extracellular matrix components (6).

Some genes involved in DN have been shown to have sex-specific effects (14;15). For example, the RANTES receptor gene (*CCR5)* is only associated with DN in men (14), and two single nucleotide polymorphisms in the podocyte slit diaphragm gene (*ACTN4*) were only associated with DN in women (15).

Therefore, we investigated whether the association between the *CNDP1* gene and DN due to type 2 diabetes is sex-specific. Furthermore, we studied whether the association between DN and the *CNDP1* gene is independent of the susceptibility for type 2 diabetes itself.

Study Design and Methods

The institutional Medical Ethics committees of the participating hospitals approved of the studies described below.

CASE GROUPS

For the first case group, female and male Caucasian diabetic patients with DN from the case-control study of Janssen *et al.* were re-assessed separately. DN was defined as diabetes with retinopathy with either macroalbuminuria or who were on dialysis (because of DN). The details of the recruitment of this cohort are described elsewhere (6). In the present analysis, only 114 DN patients with type 2 diabetes were included, and this group will be referred to as DN group 1.

For the second case group, DN patients were selected from the ZODIAC (Zwolle Outpatient Diabetes Project Integrating Available Care) study (16). DN was defined as having either an eGFR < 60 mL/min/1.73 m^2 (17) in combination with an albumin excretion above 30 mg/L (18), or macroalbuminuria (above 300 mg/L) in combination with retinopathy (19). ZODIAC was a cross-sectional, single-centre study, investigating only patients with type 2 diabetes, selected from a population of > 95% Caucasian origin. Patients were recruited from 61 general practitioners from 1998–2000. In this study, 90 DN patients were identified and these will be referred to as DN grou

For the third group, DN patients were selected out of a total of 875 patients from the NECOSAD (Netherlands Cooperative Study on the Adequacy of Dialysis) study (20). NECOSAD is a multicenter, prospective follow-up study of patients with ESRD who were included at the start of dialysis, between 1997 and 2005. For the present analysis, only patients with type 2 diabetes of Caucasian origin and ESRD due to DN were selected; 66 DN patients were included and are referred to as DN group 3.

CONTROL GROUPS

The first control group is a diabetic group, consisting of Caucasian patients with type 2 diabetes without microalbuminuria for at least 15 years, in the absence of ACE inhibitor treatment (6;16). This control group is referred to as diabetic non-nephropathy controls.

The second control group is a population control group selected from the SUNSET (Surinamese in the Netherlands, study for ethnicity and health) study, a population-based, cross-sectional survey (21). In brief, between 2001 and 2003, a random sample of non-institutionalized adults aged 35–60 years was selected. In the present study, only

the 472 white Dutch participants, of whom the genotypic distribution was described in a previous report (4), are used and referred to as population controls.

To investigate whether the association between DN and the *CNDP1* gene is not due to susceptibility for type 2 diabetes, we additionally studied 562 patients with type 2 diabetes without DN (defined as any of the criteria above) selected from the ZODIAC study participants, referred to as the type 2 diabetes population, to compare with the general population.

GENOTYPING

Genotyping was performed as described previously (4). In brief, after PCR amplification, fragment analysis was performed on the ABI-3130 analyzer to determine the number of leucine repeats in each allele. The success rate was, on average, 95% and no errors were detected. Genotyping was performed partially in Leiden and in Mannheim. Some of the samples were measured in both institutes and there was a 100% concordance.

STATISTICAL ANALYSIS

The baseline characteristics of the groups are presented as means and standard deviations or percentages. Continuous variables were tested using the student *t*-test and numeric variables using chi-square. All groups were tested for Hardy-Weinberg equilibrium (HWE), using a chi-square test.

First, the frequency of the 5-5 homozygous genotype in the respective DN groups was compared with the 93 diabetic non-nephropathy controls stratified by sex, to investigate the relevance of the genotype to disease etiology.

Second, the genotype risk for the population was estimated through comparison of the DN groups with population controls. Odds ratios with confidence intervals were calculated. A pooled analysis was performed to determine the total effect for females and males separately, combining the three case groups when compared with sex-matched population controls. The fixed-effects model (inverse variance method) was used when heterogeneity was $P > 0.1$ (chi square) and the random-effects model when heterogeneity was $P < 0.1$.

Finally, to assess whether the susceptibility for DN is independent of susceptibility for type 2 diabetes, we compared the type 2 diabetes population with population controls. The statistical analyses were all performed using SPSS 16.0 and R version 2.9.0.

Results

The baseline characteristics of the three DN groups are described in Table 1 and comparisons are made with diabetic non-nephropathy controls. Baseline characteristics did not differ between diabetic non-nephropathy controls and the type 2 diabetes population, except for diabetes duration (data not shown). There was no significant difference in 5-5 homozygous genotype frequency between the diabetic non-nephropathy controls and the population controls consisting of either all patients ($P = 0.8$), all women ($P = 0.07$), or all men ($P = 0.13$).

All cohorts were in HWE (online appendix).

Table 1. Baseline characteristics

	Diabetic nephropathy groups			T2D* > 15 years without diabetic nephropathy
	1	2	3	
Total	**n = 114**	**n = 90**	**n = 66**	**n = 93**
Age (years)	64.0 ± 11.07	73.4 ± 8.40†‡	66.2 ± 8.97	65.8 ± 11.30
Sex (n (%) male)	64 (56.1)	30 (33.3) †‡	35 (53.0)	47 (50.5)
Diabetes duration (years)	14.3 ± 8.38†	9.7 ± 9.51†‡	15.2 ± 10.99†	22.2 ± 6.78
HbA1c (%)	7.5 ± 1.71	7.5 ± 1.2	-	7.3 ± 1.51
Women				
Age (years)	64.2 ± 11.64	72.2 ± 9.09†‡	67.2 ± 7.57	65.1 ± 12.07
Diabetes duration (years)	14.8 ± 9.24*	9.2 ± 7.77†‡	15.9 ± 11.83†	22.9 ± 6.50
HbA1c (%)	8.0 ± 2.08	7.4 ± 1.12	-	7.5 ± 1.65
Men				
Age (years)	63.7 ± 10.68	75.9 ± 6.22†‡	65.3 ± 10.07	66.4 ± 10.60
Diabetes duration (years)	13.9 ± 7.81†	10.7 ± 12.4†	14.7 ± 10.39†	21.6 ± 7.05
HbA1c (%)	7.2 ± 1.32	7.6 ± 1.38	-	7.6 ± 1.38

*T2D = type 2 diabetes, † $P < 0.05$ compared to T2D > 15 years without diabetic nephropathy, ‡ $P < 0.05$ compared to either DN group 1 or 3. No significant differences were seen between group 1 and 3 or between women and men in each of the DN groups

Relevance of the 5-5 homozygous genotype to disease etiology

Overall, the 5-5 homozygous genotype frequency of the DN groups did not differ from the diabetic non-nephropathy controls or between the DN groups ($P = 0.2$). Women in all three DN groups had a significantly lower frequency of the 5-5 homozygous genotype compared with female diabetic non-nephropathy controls (Table 2), also after Bonferroni adjustment for multiple testing. In contrast, men in all three DN case groups had a higher frequency of the 5-5 homozygous genotype compared with male diabetic non-nephropathy controls.

Table 2. The relation between 5-5 homozygous genotype and DN

	Diabetic nephropathy groups			T2D*>15 years without DN
	1	2	3	
Total	**n = 114**	**n = 90**	**n = 66**	**n = 93**
Frequency 5-5 (%)	28.1	35.6	40.9	43.0
P †	0.03	0.30	0.80	
Women	**n = 50**	**n = 60**	**n = 31**	**n = 46**
Frequency 5-5 (%)	24.0	28.3	25.8	58.7
P †	0.001	0.002	0.006	
Men	**n = 64**	**n = 30**	**n = 35**	**n = 47**
Frequency 5-5 (%)	31.2	50.0	53.4	27.7
P †	0.62	0.05	0.02	

*T2D = type 2 diabetes, † $P < 0.05$ compared to T2D > 15 years without diabetic nephropathy

Genotype risk for the population

No heterogeneity was detected in women ($P = 0.64$), but heterogeneity was detected in men ($P = 0.09$). The three DN groups were pooled for women and men separately, resulting in a genotype risk of 0.5 [0.30–0.68] in women and 1.2 [0.77–1.69] in men (Figure 1).

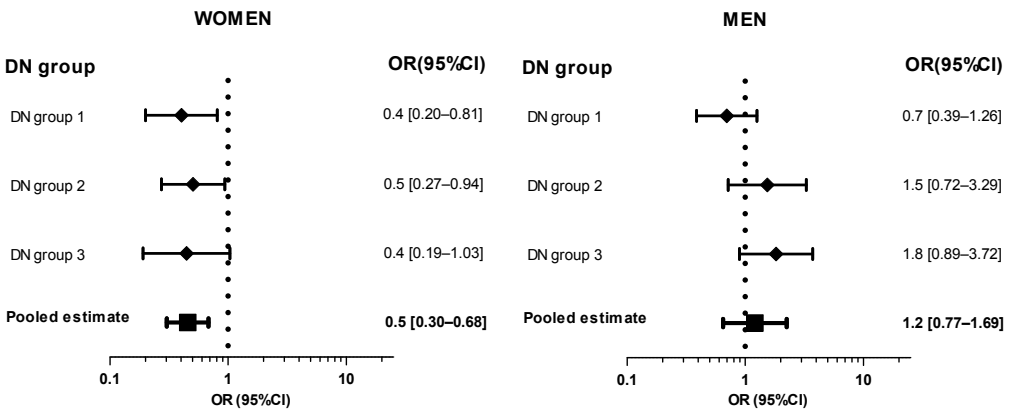

Figure 1. The relationship between the 5-5 homozygous *CNDP1* genotype and diabetic nephropathy in women (upper panel) and men (lower panel) in the three independent diabetic nephropathy groups, and a pooled analysis (total) compared with population controls.

Specificity of 5-5 homozygous genotype for DN, not type 2 diabetes

The 5-5 homozygous genotype frequency of both the 562 type 2 diabetes population and the 472 population controls are shown in Table 3, showing similar frequency of the 5-5 homozygous genotype.

Table 3. Comparison between type 2 diabetes population and population controls

	Type 2 diabetes population	**Population controls**	
Total	**n = 562**	**n = 472**	***P***
Frequency 5-5 (%)	38.1	41.7	0.23
Women	**n = 319**	**n = 239**	
Frequency 5-5 (%)	39.5	43.9	0.23
Men	**n = 243**	**n =233**	
Frequency 5-5 (%)	36.7	39.5	0.58

Discussion

Our results show a sex-specific effect of the *CNDP1* genotype in relation to DN, suggesting that the 5-5 homozygous genotype is only protective in women. The frequency of the 5-5 homozygous genotype was determined in three independent DN groups. These groups were compared with two control groups; patients with type 2 diabetes and a low risk of ever developing DN and a sample from the general population. Compared with the diabetic control group, the 5-5 homozygous genotype frequency was significantly lower in women with DN in all three cohorts, but not in men. The population control group serves to estimate the genotype risk for the population, showing that women with the 5-5 homozygous genotype have a 2-fold reduced risk of ever developing DN. Furthermore, this study shows similar frequencies of the 5-5 homozygous genotype in a large type 2 diabetes population and the general population, underlining that the association with DN is independent of a genetic susceptibility for type 2 diabetes.

The 5-5 homozygous genotype leads to lower carnosinase activity compared with the other genotypes (4;6), leaving more carnosine free to protect the kidney from oxidative stress. Since men have higher carnosine levels in their muscle tissue and women have slightly higher serum carnosinase levels (22), differences in carnosinase activity due to the different *CNDP1* polymorphisms may have a stronger impact in women. Carnosine content in the muscles of female mice was shown to increase after testosterone administration, the increase was 268% (23). This might be because carnosine synthetase,

the enzyme that synthesizes carnosine, is upregulated by testosterone. It is possible that this phenomenon plays a role in DN, because both carnosine synthetase (preliminary results) and androgen receptors are expressed in human kidney (24).

Another explanation for the sex-specific effect found in this study is that the association between the *CNDP1* gene and DN is lost in men due to selective survival by cardiovascular disease. As carnosine has shown to be protective against oxidative stress and hemodynamic damage (6;12;13), this might also explain its role in cardiovascular death in DN patients. Men with DN due to type 2 diabetes have a higher risk for cardiovascular disease than women (25). Therefore this might be more prominent in men. Further support for this theory comes from the ZODIAC study. Men with a diabetes duration < 10 years and the 5-5 homozygous genotype have a significantly lower mortality risk due to cardiovascular disease than patients with more than 10 leucine repeats in the *CNDP1* genotype (data not shown). We found no difference in cardiovascular death between the different genotypes in women.

The relatively older age of DN group 2 might influence the number of subjects with an eGFR < 60 ml/min/1.73m^2. Therefore, a sensitivity analysis was performed, adopting increasingly stringent definitions of DN. The results of this analysis support the conclusion that the 5-5 homozygous genotype is protective in women (online appendix).

The statistical power to detect a similar association as was seen in women in men ranged from 97- 100% within the three DN groups. Insufficient statistical power therefore does not explain the sex-specific effect found in this study.

Limitations of this study are that ethnic origin is not defined by ethnic markers in these Caucasian populations and that sample sizes are relatively small. We performed a sensitivity analysis to exclude population stratification and a permutation analysis to rule out that our results are due to random fluctuation. These analyses support that population stratification or chance are unlikely to explain the sex-specific effect found in this study (online appendix).

Another limitation is that the three DN groups are compared to the same control group.

In conclusion, this study shows a sex-specific effect of the association between the *CNDP1* gene and diabetic nephropathy in three independent patient groups with diabetic nephropathy due to type 2 diabetes, with women being protected by the 5-5 homozygous genotype.

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Disclosure

None of the authors have conflicts of interest to disclose.

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6

CNDP1 POLYMORPHISM PREDISPOSES FOR PROGRESSION TO END STAGE RENAL DISEASE AFTER GLOMERULAR DAMAGE

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Submitted

Abstract

A polymorphism in the number of CTG-repeats in exon 2 of the *CNDP1* (carnosinase) gene correlates with the development of nephropathy in diabetes patients. Carnosinase degrades carnosine, which protects glomeruli against oxidative and hemodynamic damage. This study investigated whether the *CNDP1* polymorphism is associated with occurrence of ESRD and mortality risk in patients with vascular kidney diseases other than diabetic nephropathy, such as renal vascular disease and chronic glomerulonephritis.

Included were 97 Caucasian end stage chronic glomerulonephritis patients, 143 end stage renal vascular disease patients and 732 healthy Caucasian controls. Furthermore, 104 end stage polycystic kidney disease patients and 95 end stage tubulointerstitial nephritis were included as disease controls. Prevalence of genotypes was compared by calculating odds-ratios, survival of patients by Kaplan-Meier techniques.

Compared to patients with genotypes resulting in lower carnosinase activity, genotypes with higher carnosinase activity were associated with an increased risk of developing ESRD in patients with renal vascular disease (OR [95% CI] 2.47 [1.02, 5.98]) and chronic glomerulonephritis (5.08 [2.15, 11.99]). These chronic glomerulonephritis patients also had an increased mortality risk (p log rank 0.01). In patients with other primary kidney diseases the genotype was not associated with ESRD or mortality.

In conclusion, in patients with glomerular damage the *CNDP1* polymorphism predisposes for development of ESRD. In chronic glomerulonephritis this was also reflected by increased mortality. These findings support the hypothesis that there is a common genetic basis for progression to ESRD after glomerular damage.

Introduction

End stage renal disease (ESRD) has reached epidemic proportions and the number of ESRD patients is still increasing [1]. These patients require renal function replacement therapy by either dialysis or transplantation. This leads to loss of quality of life, high mortality rates and has become a great economic burden [1]. Identifying risk factors for progression to ESRD are therefore of great interest and might be able to prevent susceptible individuals from developing ESRD by earlier and more aggressive treatment. One of the most important risk factors for developing ESRD seems to be a positive family history of ESRD [2]. Lei *et al.* found that familial aggregation of renal disease could not be fully explained by familial clustering of diabetes and hypertension. Therefore it is likely that a separate genetic susceptibility factor exists for progression of ESRD [3].

A possible susceptibility locus for diabetic nephropathy on chromosome 18q has been identified in earlier studies [4;5]. We identified the responsible modifying gene within this locus as the *CNDP1* gene [6]. These findings were further confirmed in 963 American patients of European descent with type 2 diabetes-induced nephropathy [7], but not in patients with type 1 diabetes [8]. Type 2 diabetes patients with the homozygosity for the Mannheim allele (5 copies of a trinucleotide repeat encoding for leucine in the leader peptide on exon 2) of the *CNDP1* gene demonstrated a 2.56-fold reduced risk for developing diabetic nephropathy (DN) compared to individuals with more leucine repeats (6-8 repeats) [6]. The presence of more than 5 leucine repeats has been shown to lead to higher serum carnosinase secretion [9] and more serum carnosinase activity [6;10]. Serum carnosinase is known to degrade histidine-containing dipeptides called carnosines, which function as scavengers of reactive oxygen species [11] and as inhibitors of angiotensin converting enzyme (ACE) [12].

Since injury to glomerular cells by oxidative stress and hemodynamic factors is not confined to development of diabetic nephropathy, we hypothesize that lower number of leucine repeats in the *CNDP1* play a protective role in progression to ESRD in underlying renal diseases due to glomerular and microvascular injury, such as chronic glomerulonephritis and renal vascular disease. In line with this hypothesis, one would expect that within these vascular disease groups, survival would differ in patients with different *CNDP1* genotypes.

Methods

Patient and control subjects

Patients were selected from the Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD), a multicenter, prospective follow-up study of ESRD patients who were included at the time of the initiation of dialysis [13]. Data on ethnic background, gender, primary kidney disease, comorbidities, and modality were collected between four weeks prior and two weeks after the start of dialysis. Patients were at least 18 years of age with no previous renal replacement therapy and were followed till death or censoring. Reasons for censoring included transplantation, recovery of renal function or loss to follow-up. All local medical ethics committees approved of the study and patients gave informed consent before inclusion.

For the current study, data from chronic glomerulonephritis, polycystic kidney disease, renal vascular disease and tubulointerstitial nephritis were selected. These diseases were classified according to the European Renal Association-European Dialysis and Transplantation Association (ERA-EDTA) codes (http://www.era-edta-reg.org/files/annualreports/pdf/AnnRep2006.pdf.). Based on these ERA-EDTA codes, primary kidney disease groups were defined: chronic glomerulonephritis (cGN, codes 9 to 20), tubulointerstitial nephritis (TIN, codes 20 to 40), polycystic kidney disease (PKD, codes 40 to 50) and renal vascular disease (RVD, codes 70 to 73, or code 79). Patients that were not of Caucasian origin were excluded, as the distribution of *CNDP1* is dependent of ethnic origin [10]. Comorbidity was defined according to the risk criteria of Khan *et al.* [14]. The Khan index is a combination of age and co-morbidity that divides risk groups into three degrees of severity as low, medium, or high. Three months after beginning dialysis, a blood sample and 24-hour urine sample were collected on the same day. Serum albumin, plasma creatinine, and plasma urea levels were determined. Urea and creatinine levels were also analyzed in the urine sample. Genomic DNA was isolated from peripheral blood with the Puregene® DNA isolation kit (Gentra, Minneapolis, USA). The genotype frequencies in NECOSAD patients were compared to those in 732 healthy controls above 18 years of age. To eliminate ethnic differences, we only included Caucasian subjects who confirmed that all grandparents were of Northwest European origin.

Genotyping

CNDP1 genotyping was performed as described elsewhere [6]. In brief, a standard PCR protocol was used with the primers GCGGGGAGGGTGAGGAGAAC (forward) and GGTAACAGACCTTCTTGAGGAATTTGG (reverse). The denaturating, annealing and extension temperatures were 94°C, 60°C, and 72°C, respectively. Fragment analysis was performed on the ABI-3130 analyzer (Perkin Elmer) to determine the number of leucine repeats on each allele. A product length of 157, 160, and 163 bp corresponded to 5, 6, or 7 CTG (Leu) codons, respectively.

Determination of serum-carnosinase activity

Serum-carnosinase activity was determined according to the method described by Teufel *et al*. [15]. Briefly, the reaction was initiated by addition of substrate (L-carnosine) to a serum sample and stopped after 10 minutes of incubation at 30°C by adding 1% trichloracetic acid. Liberated histidine was derivatized with *o*-phtaldialdehyde (OPA). Fluorescence was measured by excitation at 360 nm and emission at 460 nm. Serum samples were obtained from 60 healthy controls.

Mortality

Causes of death were determined by treating physicians and classified according to the codes of the ERA-EDTA which can be found at: http://www.era-edta-reg.org/files/annualreports/pdf/AnnRep2006.pdf. The following codes were classified as cardiovascular mortality: 0 (cause of death uncertain/not determined), 11 (myocardial ischemia and infarction), 14 (other causes of cardiac failure), 15 (cardiac arrest, cause unknown), 18 (fluid overload), 22 (cerebrovascular accident), 26 (hemorrhage from ruptured vascular aneurysm, not code 22 or 23), or 29 (mesenteric infarction).

Statistical analysis

In all analyses subjects carrying the *CNDP1* Mannheim variant were used as reference group and contrasted to all other possible genotypes (5-0, 5-6, 5-7/6-6, 6-7/6-8/7-7). Hardy-Weinberg equilibrium was calculated using the gene-counting method, and differences between NECOSAD patients and the control group were assessed by chi-square test. In NECOSAD patients, differences in baseline characteristics between the different *CNDP1* genotype groups were tested with the chi-square test for dichotomous and categorical variables and analysis of variance for continuous variables. The Armitage

Trend Test was used to test for genetic association, as the genotypes could be ranked according to the respective enzyme activities.

Survival of patients with different *CNDP1* genotypes was analyzed by means of Kaplan-Meier survival curves. Log rank tests were used to determine survival differences. All statistical analyses were performed with SPSS statistical software (version 12; SPSS, Chicago, IL) and SAS (Version 9.1; SAS, Heidelberg, Germany).

Results

A total of 439 dialysis patients were selected from the NECOSAD, consisting of 97 chronic glomerulonephritis patients, 143 renal vascular disease patients, 104 polycystic kidney disease patients and 96 tubulointerstitial nephritis patients.

Baseline characteristics of these 439 patients and these patients grouped by allelic variant of *CNDP1* are seen in Table 1.

Carnosinase activity

The carnosinase activity was measured in 60 healthy controls with genotypes 5-5, 5-6, 6-6, 5-7. The genotype 5-0 was estimated on theoretical enzyme activity and the 5-8 was extrapolated from a different study done by Riedl *et al.* [9]. A clear correlation was found between carnosinase enzyme levels and the number of leucine repeats in the *CNDP1* gene. The various genotypes were ranked according to the corresponding enzyme activities (Figure 1).

Table 1. Baseline characteristics of patients under study (N=439), grouped by allelic variant of *CNDP1*

	Total	***CNDP1* genotype**			
		5-5	**5-6**	**5-7/6-6**	**6-7/6-8/7-7**
	N=439	**N=163**	**N=164**	**N=84**	**N=28**
Age (yrs)	59.5 (14.0)	59.7 (13.8)	59.2 (13.9)	59.0 (15.0)	61.6 (14.3)
Gender (% female)	35.1	38.7	32.3	32.1	39.3
Chronic Therapy (% HD)	62.9	65.0	59.8	67.9	53.6
Primary kidney disease (%)					
Glomerulonephritis	22.1	17.2	22.0	27.4	35.7
Interstitial nephritis	21.6	27.6	17.7	15.5	28.6
Polycystic kidney disease	23.7	27.0	24.4	21.4	7.1
Renal vascular disease	32.6	28.2	36.0	35.7	28.6
Khan co-morbidity score (%)					
low	49.7	51.5	50.0	48.8	39.3
moderate	28.2	30.7	28.0	23.8	28.6
high	22.1	17.8	22.0	27.4	32.1
DM co-morbidity (%)	5.1	4.4	4.9	6.0	7.1
CVD co-morbidity (%)	35.3	31.0	36.5	36.8	48.1
Smoking habit (%)					
never	27.1	27.6	28.7	28.6	10.7
ever, >3 mo ago	43.1	45.4	41.5	41.7	42.9
ever, <=3 mo ago	4.8	3.7	5.5	3.6	10.7
current	25.1	23.3	24.4	26.2	35.7
GFR (ml/min/1.73m^2)†	5.3 (3.1)	5.3 (2.7)	5.4 (3.4)	5.3 (3.1)	5.1 (3.5)
Blood pressure (mmHg)					
systolic	149.1 (24.8)	149.6 (26.2)	147.6 (24.7)	151.6 (24.1)	147.3 (19.8)
diastolic	83.6 (12.8)	84.0 (12.8)	82.5 (13.1)	84.8 (13.2)	83.2 (10.7)

Mean values ± SD are given for continuous variables. For categorical variables percentages are shown. DM, diabetes mellitus; GFR, glomerular filtration rate; CVD, cardiovascular disease; *Exclusive vasculitis; †GFR = glomerular filtration rate, measured between four weeks prior to and two weeks after start of dialysis (for 5-5 [N=91], 5-6 [N=100], 5-7/6-6 [N=48] and 6-7/6-8/7-7 [N=19]).

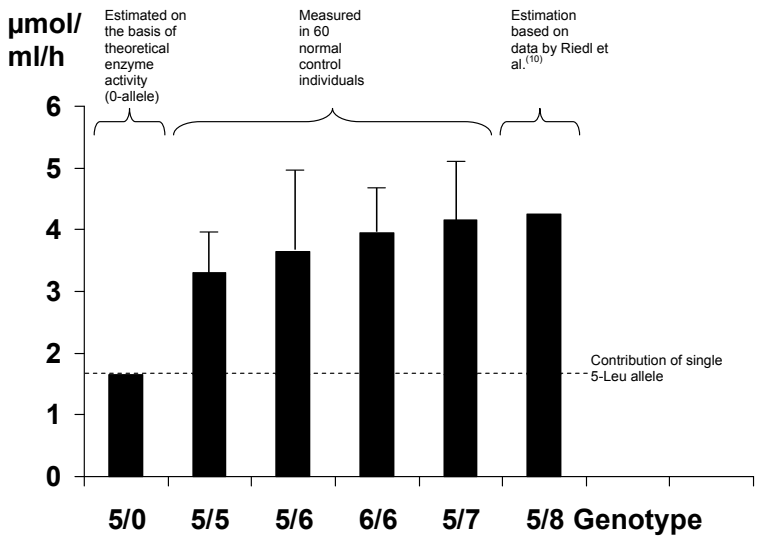

Figure 1. Estimated and measured serum-carnosinase activity in compound heterozygosity with the Mannheim allele (5-Leu) in normal Caucasian individuals.

Analysis of CNDP1 genotype frequencies by comparison of disease subgroups. Patients with chronic glomerulonephritis had significantly more leucine repeats than controls (Table 1, p= 0.0006). ESRD patients with the highest number of leucine repeats in the *CNDP1* gene have a 5.1 (95% CI 2.15, 11.99) higher risk of developing ESRD due to glomerulonephritis compared to glomerulonephritis patients with lower number of leucine repeats (Table 2). The renal vascular disease patient group also showed significant association with *CNDP1* repeat length (p=0.011) (Table 1). ESRD patients with the highest number of leucine repeats in the *CNDP1* gene have a 2.5 (95% CI 1.02, 5.98) higher risk of developing ESRD due to renal vascular disease compared to renal vascular disease patients with lower number of leucine repeats (Table 2). Patients who became dialysis-dependent due to polycystic kidney disease or tubulointerstitial nephritis, as well as normal controls, all had similar distributions of the *CNDP1* genotypes (Table 2).

Table 2. Distribution of *CNDP1* leucine repeats of NECOSAD patients grouped by primary kidney disease compared to controls.

	NECOSAD grouped by primary kidney disease									
	cGN		RVD		TIN		PKD		Control group	
CNDP1[#]	(n=97)		(n=143)		(n=95)		(n=104)		(n=732)	
	n	%	n	%	n	%	n	%	n	%
5/0, 6/0, 7/0	0	0	0	0	0	0	0	0	13	1.8
5-5	28	28.9	46	32.2	45	47.4	44	42.3	270	36.8
5-6	36	37.1	59	41.3	29	30.5	40	38.5	303	41.4
5/7, 6/6	23	23.7	30	21.0	13	13.7	18	17.3	127	17.3
6/7, 6/8, 7/7, 7/8	10	10.3	8	5.6	8	8.4	2	1.9	19	2.6
p*	**0.0006**		**0.011**		0.55		0.74			

GN indicates chronic glomerulonephritis; TIN, tubulointerstitial nephritis; PKD, polycystic kidney disease; RVD, renal vascular disease; DN, diabetic nephropathy; and OMD, other multisystem diseases.
*) NECOSAD patients vs. the control group as determined with Armitage Trend Test.
#) Genotypes grouped according to enzyme activity (Table 1).

Mortality

Survival probabilities for patients with different *CNDP1* genotypes were not different (log rank test, p=0.62). A stratified survival analysis revealed that the survival probability in patients with glomerulonephritis (log rank test, p<0.01) as primary renal disease were significantly different between the leucine repeat groups (5-5, 5-6, 5-7/6-6, 6-7/6-8/7-7) (Figure 2). Patients with glomerulonephritis and a high number of leucine repeats had higher mortality rate compared to glomerulonephritis patients with lower number of leucine repeats. Survival probabilities in ESRD patients due to polycystic kidney disease, tubulointerstitial nephritis and renal vascular disease did not differ between the leucine repeat groups.

Table 3. Genotypic odds ratios (OR) comparing genotype-related risks to the 5Leu / 5Leu reference and primary causes of development of ESRD.

CNDP1	cGN (OR, 95% CI)	RVD (OR, 95% CI)
5/5 (ref.)	1.00	1.00
5/6	1.14 (0.68, 1.92)	1.14 (0.75, 1.73)
5/7, 6/6	1.70 (0.95, 3.08)	1.35 (0.82, 2.25)
6/7, 6/8, 7/7, 7/8	**5.08 (2.15, 11.99)**	**2.47 (1.02, 5.98)**

cGN: chronic glomerulonephritis; RVD: renal vascular disease.

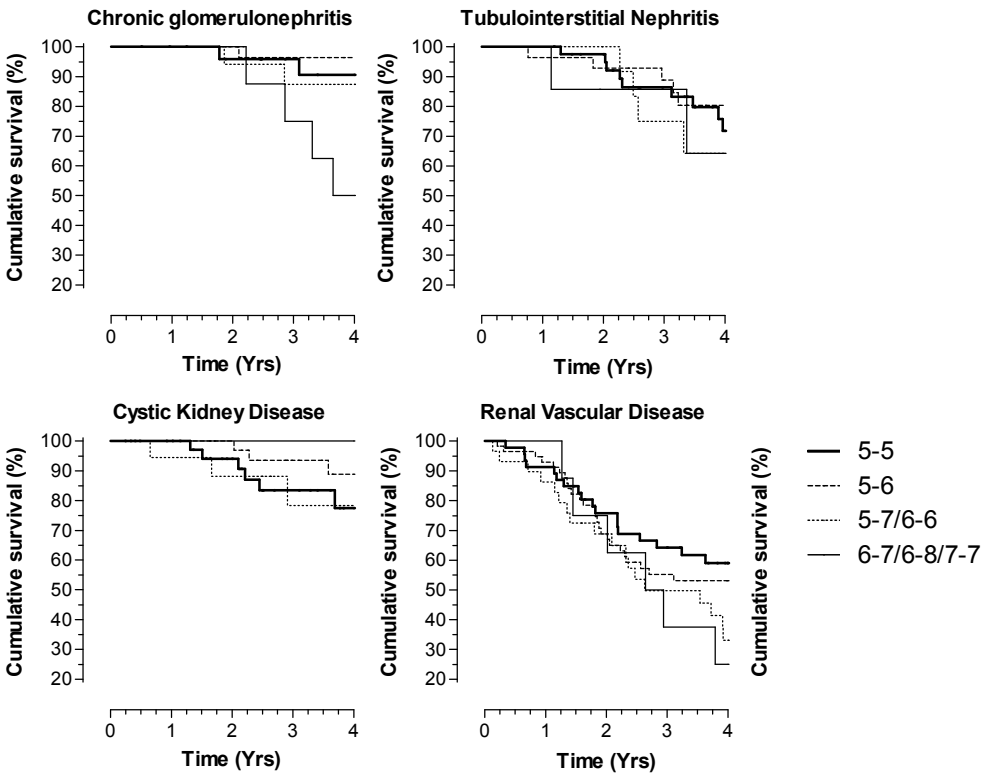

Figure 2. Survival within the first four years of follow-up of patients with chronic glomerulonephrits, tubulointerstitial nephritis, cystic kidney disease and renal vascular disease.

Discussion

Our results demonstrate that the higher number of leucine repeats in the *CNDP1* genotype is related to a faster progression to ESRD in patients with compromised kidney function due to chronic glomerular inflammatory renal diseases and the group of patients with renal vascular disease. In line with these results, the mortality risk is increased in chronic glomerulonephritis patients with higher number of leucine repeats in the *CNDP1* gene, which is associated with higher serum carnosinase levels. Our data show a correlation between the leucine repeat distribution of the *CNDP1* gene and renal vascular disease, however there was no relation with survival. As predicted, patients who developed ESRD due to either polycystic kidney disease or tubulointerstitial had a *CNDP1* genotype

distribution similar to that of healthy controls. These findings support the hypothesis that the leucine repeat in *CNDP1* may contribute to microvascular damage.

In chronic glomerulonephritis the higher number of leucine repeats in the *CNDP1* gene seems to be involved in progression to ESRD and mortality on dialysis. There are two pathways by which this association can be explained. Common to the development of ESRD in these patients is that the development of progressive glomerulosclerosis is accompanied by oxidative injury to glomerular cells from reactive oxygen species (ROS) and hemodynamic factors [16]. Histidine-containing dipeptides such as carnosines reportedly function as ROS scavengers [17], natural inhibitors of transforming growth factor beta (TGF-beta) production [6], anti-apoptotic compounds [18], and natural inhibitors of ACE [12]. The rapid degradation of these dipeptides by locally produced serum-carnosinase may impair a protective mechanism required for recovery after renal disease. Indeed, transfection experiments have shown that multiple leucine repeats in carnosinase results in increased secretion of the enzyme [9] and our results show an increased carnosinase activity with increasing number of leucine repeats. This current result is a replication, as this was found in both Caucasians [6] and South Asians [10]. Furthermore, carnosinase was expressed in the kidney, making a specific role in the kidney likely [10]. Carnosines are released from skeletal muscle after physical exercise [19], and the beneficial effects of exercise on diabetic nephropathy [20], hypertension-induced nephrosclerosis [21;22], and progressive renal disease in general [23] have been well documented. Thus, the protective effects of carnosine seem to be essential for natural recovery of the kidney after microvascular injury.

The second possibility regards the connection between the autonomic nervous system and the kidney. Homocarnosine, a particular substrate of serum carnosinase, is composed of gamma-amino-butyric acid-L-histidine (GABA-his) [24]. Cleavage of homocarnosine by serum-carnosinase releases the neurotransmitter GABA, resulting in GABA-receptor-mediated activation of the sympathetic innervations of the kidney [24;25]. In addition, carnosine has been shown to inhibit sympathetic nerve activity directly, resulting in reduction of systemic blood pressure [26]. The same research group also demonstrated the involvement of carnosine in the regulation of blood glucose levels via autonomic nerves [27]. Indeed, hyperactivity of the sympathetic nerves in both hypertension-induced renal insufficiency and progressive renal disease in general has been reported in both experimental settings and clinical studies [28-31].

Recently, we found that the relation between the *CNDP1* gene and DN is sex-specific, including patients with DN from the NECOSAD study [32]. We also stratified the data of this study for men and women. The frequency of the 5-5 homozygous genotype was lower in women than in men in chronic glomerulonephritis patients (23.1% vs. 31.0%) and renal vascular disease patients (29.7% vs. 33.0%). Although this is in line with the data in DN, no definite conclusion should be drawn as due to the stratification the sample size became too small.

Ideally, one would have preferred to compare the ESRD group with the diseased patients not progressing to ESRD to exclude the possible correlation between susceptibility for the disease. However we feel that such correlation is unlikely because of the aetiological heterogeneity within the disease groups. Furthermore, the different diseases can be seen to serve as disease control groups.

Our data have clinical implications. Carnosine and related compounds have already been used as therapies for cataracts [33] and gastric ulcers [34]. In rats induced with gentamicin, treatment with carnosine led to a significant improvement of the kidney function [35]. Furthermore, gentamicin-induced glomerular shrinkage was absent in those rats treated with carnosine [35]. In humans, carnosine seems to be hydrolyzed within 2 hours after carnosine is absorbed by the intestine and enters the bloodstream [36]. However, when Parkinson patients were treated with carnosine in addition to dopamine (L-dopa), they had a decreased level of protein carbonyls in their blood plasma and increased levels of red cell superoxide dismutase compared with patients treated with only dopamine (L-dopa) [37]. This suggests that supplementation with carnosine has a biochemical effect after oral intake and could, therefore, be of potential therapeutic value. Our study indicates that carnosine supplementation can also be considered in patients with chronic glomerulonephritis and renal vascular disease.

In summary, here we have provided evidence for a genetic predisposition for progression to ESRD due to chronic glomerulonephritis and renal vascular disease. In glomerulonephritis this was reflected in both genotype distribution and in mortality risk. Hereby we identified a genetic polymorphism that seems to be involved in the clinical course of renal disease after sustained glomerular injury.

Disclosure

I declare that none of the authors have conflicts of interest.

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7

VEGETARIANISM, FEMALE GENDER AND INCREASING AGE, BUT NOT *CNDP1* GENOTYPE, ARE ASSOCIATED WITH REDUCED MUSCLE CARNOSINE LEVELS IN HUMANS

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Abstract

Carnosine is found in high concentrations in skeletal muscles, where it is involved in several physiological functions. The muscle carnosine content measured within a population can vary by a factor 4. The aim of this study was to further characterize suggested determinants of the muscle carnosine content (diet, gender and age) and to identify new determinants (plasma carnosinase activity and testosterone).

We investigated a group of 149 healthy subjects, which consisted of 94 men (12 vegetarians) and 55 women. Muscle carnosine was quantified in M. soleus, gastrocnemius and tibialis anterior using magnetic resonance proton spectroscopy and blood samples were collected to determine *CNDP1* genotype, plasma carnosinase activity and testosterone concentrations.

Compared to women, men have 36%, 28% and 82% higher carnosine concentrations in M. soleus, gastrocnemius and tibialis anterior muscle respectively, whereas circulating testosterone concentrations were unrelated to muscle carnosine levels in healthy men. The carnosine content of the M. soleus is negatively related to the subjects' age. Vegetarians have a lower carnosine content of 26% in gastrocnemius compared to omnivores. In contrast, there is no difference in muscle carnosine content between subjects with a high or low ingestion of beta-alanine within an omnivore diet. Muscle carnosine levels are not related to the polymorphism of the *CNDP1* gene nor to the enzymatic activity of the plasma carnosinase.

In conclusion, neither *CNDP1* genotype, nor the normal variation in circulating testosterone levels affect the muscular carnosine content, whereas vegetarianism, female gender and increasing age are factors associated with reduced muscle carnosine stores.

Introduction

Carnosine (β-alanyl-L-histidine) is a dipeptide found in high concentrations in skeletal muscles. Several of carnosine's physiological actions are relevant to muscular function and homeostasis, such as pH buffering (1; 2; 5; 6), antioxidant effects (6; 24), increasing the Ca^{2+} sensitivity of the contractile apparatus (12; 25) and inhibiting protein glycation (19), as recently reviewed (6; 8; 37). Interestingly, recent studies have shown that elevated muscle carnosine content is associated with attenuated fatigue and improved exercise performance in humans (10; 18; 35; 40; 41; 44; 48). The carnosine content in human muscles usually amounts to 20-30 $mmol \cdot kg^{-1}$ in dry weight (5-8 mM in wet weight), but can easily vary by a factor 3-4 between the highest and the lowest concentrations measured within a population. Yet, the muscle carnosine content is rather constant, as we showed that intra-individual variation in muscle carnosine content is only 9-15% over a 3-month period (3). The MRS-based technique (10) gives us the opportunity to explore, without the need for muscle biopsy, existing and new determinants of the variation in muscle carnosine content within a large population.

The most established determinant of the muscle carnosine content is muscle fiber type. HPLC-based single fiber analysis in humans showed a 30-100% higher carnosine content in fast-twitch muscle fibers in comparison with slow-twitch (15; 18; 23). Indeed, Mannion *et al.* (27) and Suzuki *et al.* (43) showed a positive correlation between muscle fiber type and muscle carnosine content. Furthermore, elite sprinters who are characterized by a high proportion of fast-twitch muscle fibers have a higher muscle carnosine content in comparison with marathon runners (32).

The amount of food from animal sources is a likely determinant of muscle carnosine levels since beta-alanine, the rate-limiting precursor in carnosine synthesis, is exclusively found in meat and fish. The ingestion of very high doses of beta-alanine in pure form (4-6.4 $g \cdot day^{-1}$ as a food supplement) for several weeks (4-10 wks) results in 40-80% increases in muscle carnosine content (3; 10; 17; 18; 23). Whether variations in daily meat intake within a normal omnivorous diet also affect muscular carnosine content, remains to be established. The chronic and complete withdrawal of dietary beta-alanine, such as in vegetarianism, supposedly results in decreased carnosine content, although the current evidence is scarce (16).

Men have been shown to have higher (21%) carnosine content in the quadriceps femoris when compared to women (26). This sexual dimorphism is more pronounced in mice with a male/female ratio of approximately 3.5/1 (33), but absent in horses (28). Concerning the effect of age, several studies on rodents demonstrated a decreasing muscle carnosine content of 35-50% with senescence (9; 21; 42). To our knowledge, no longitudinal studies on humans are available, but there is cross-sectional evidence for a decreased muscle carnosine content of 33-60% in elderly people with specific pathologies (42; 45).

A possible explanation for a lower muscle carnosine content amongst elderly people and women is their lower plasma (free) testosterone content. Both cross-sectional and longitudinal studies have confirmed an age-associated decline of plasma testosterone in aging men (reviewed in (22). Penafiel *et al.* (33) hypothesized that androgens might up regulate carnosine synthase, based upon the findings that the muscle carnosine content was reduced by 40% in castrated mice and that testosterone injections increased muscle carnosine content by 268% in female mice. To our knowledge, no study has investigated a possible connection between circulating testosterone and muscle carnosine content in eugonadal men.

Polymorphism in the enzymes involved in the synthesis (carnosine synthase) and hydrolysis (carnosinase) of the dipeptide could also contribute to the muscle carnosine content. Since the highest activity of carnosine synthase is found in skeletal muscles (6), polymorphisms of the gene encoding carnosine synthase are likely to clarify variations in carnosine. As the gene has only recently been identified (11), there are no studies that have examined the effects. A leucine repeat in exon 2 of the *CNDP1* gene, coding for the serum carnosinase enzyme, has been shown to affect carnosinase activity (20; 29) and likely the duration of the presence of carnosine in plasma. The enzyme carnosinase is supposedly not present and/or not active in skeletal muscles (3), but it is reasonable to assume that the plasma carnosinase activity could indirectly affect the muscle carnosine content.

The aim of this study was to further characterize previously reported determinants (diet, gender and age) and to identify new potential determinants (plasma carnosinase activity, *CNDP1* genotype and testosterone) of the human muscle carnosine content. Carnosine was quantified using proton magnetic resonance spectroscopy (^{1}H-MRS), as previously described (3; 10; 31), in the slow-twitch soleus and tibialis anterior and the fast-twitch gastrocnemius muscle, in order to explore the possible interaction of these factors with the muscle fiber type.

Materials and methods

Subjects. As depicted in figure 1, a total of 149 healthy subjects volunteered to participate in this study. The muscle carnosine content of 12 male vegetarians was determined. Vegetarian subjects were either lacto-ovo, or vegan and were required to be vegetarian for a minimum of 8 years (mean ± SD: 15 ± 9.5 yr; range 8-36 yr) prior to the study. Blood samples were obtained from all omnivores (82 males, 55 females) for determination of plasma carnosinase activity and *CNDP1* genotype (Age: mean ± SD: 23.9 ± 7.0, range: 18 - 69 yrs). The muscle carnosine content (38 males, 20 females) and plasma concentration of androgens (38 males) were measured in a subgroup of omnivores, of which 29 male individuals registered their meat and fish consumption during 2 weeks. The mean (± SD) age of the male population (23.9 ± 7.2 yrs, range: 19 - 47 yrs) is lower than the age of the vegetarians (31.3 ± 4.2 yrs, range: 22 - 38 yrs) but not different from the female group (23.8 ± 6.7 yrs, range: 19 - 46 yrs). The data of 19 male omnivore subjects are originating from a previous study (3). The study protocols were approved by the local Ethical Committee (Ghent University Hospital, Belgium) and written informed consent was obtained from all participants prior to the study.

^{1}H-MRS. The carnosine content of 3 skeletal muscles of the lower leg (soleus, gastrocnemius and tibialis anterior) of a subgroup of 70 subjects was measured using ^{1}H-MRS, as previously described (3). The subjects lay in supine position on their back and the lower leg was fixed in a holder with the angle of the ankle at 20° plantar flexion. All the MRS measurements were performed on a 3 Tesla whole body MRI scanner (Siemens Trio, Erlangen) equipped with a knee-coil. Single voxel point-resolved spectroscopy with the following parameters was used: repetition time (TR)= 2000 ms, echo time (TE) = 30 ms, number of excitations = 128, 1024 data points, spectral bandwidth of 1200Hz, and a total acquisition time of 4.24min. The average voxel size of the soleus, gastrocnemius lateralis and tibialis anterior was respectively 40 mm x 12 mm x 29 mm, 40 mm x 12 mm x 29 mm, 40 mm x 14 mm x 27 mm. Following shimming procedures, the linewidth of the water signal was on average 24.4 Hz, 25.5 Hz and 27.6 Hz for soleus, gastrocnemius and tibialis anterior, respectively. A 500-ml spherical container filled with an aqueous solution of 20 mM carnosine (Sigma-Aldrich) was used as an external reference for absolute quantification. The following equation was used to determine the concentration of C2-H (at ~8 ppm) carnosine in vivo:

$$[C_m] = [C_r] \cdot (S_m \cdot V_r \cdot C_{T1r} \cdot C_{T2r} \cdot T_m) / (S_r \cdot V_m \cdot C_{T1m} \cdot C_{T2m} \cdot T_r)$$

Where $[C_m]$ is the carnosine concentration in vivo; $[C_r]$ is the concentration of the external reference phantom (20 mM); S_m and S_r are the estimated signal peak areas of the muscle and reference phantom, respectively, obtained by curve fitting performed in the frequency domain and were also corrected for differences in coil loading between phantom and the muscle; corrected for V_m and V_r, the volumes of the voxels in vivo and in the phantom, respectively; C_{T1m}, C_{T2m}, C_{T1r} and C_{T2r} are correction factors for the T1 and T2 relaxation times in vivo and in the phantom, respectively; T_m and T_r are the temperatures in vivo and in the phantom, respectively. The T1 and T2 relaxation times of in vitro carnosine were measured and were found to be 2616 ± 20 ms and 250 ± 29 ms, respectively. The formulae used to calculate the correction factors are:

C_{T1} = (1-e^(-TR/T1))

C_{T2} = e^(-TE/T2)

For the determination of T1 and T2 relaxation times in vivo 5 healthy subjects (2 males and 3 females; age: 21 to 26 yr) were scanned for the soleus, 5 (3 males and 2 females; age: 21 to 25 yr) for the gastrocnemius and 5 for the tibialis anterior muscle (5 females; 22 to 26 yr). T1 was measured using a TE of 30 ms and TR was set to 1500, 2000, 2500, 3000, 3500, 4000, 5000 and 6000 ms. T2 was measured using a TR of 4000 ms and TE was set to 30, 60, 90, 120, 150 and 200 ms for soleus, 30, 45, 60, 75, 90 and 105 ms for gastrocnemius and 30, 45, 50, 60, 70 and 75 for tibialis anterior muscle. For each measurement 128 averages were acquired. The T1 relaxation times were shown to be 1488 ± 377 ms, 1771 ± 225 ms and 1692 ± 432 ms and T2 relaxation times were 152 ± 28 ms, 106 ± 50 ms an 64 ± 32 ms in soleus, gastrocnemius and tibialis anterior, respectively.

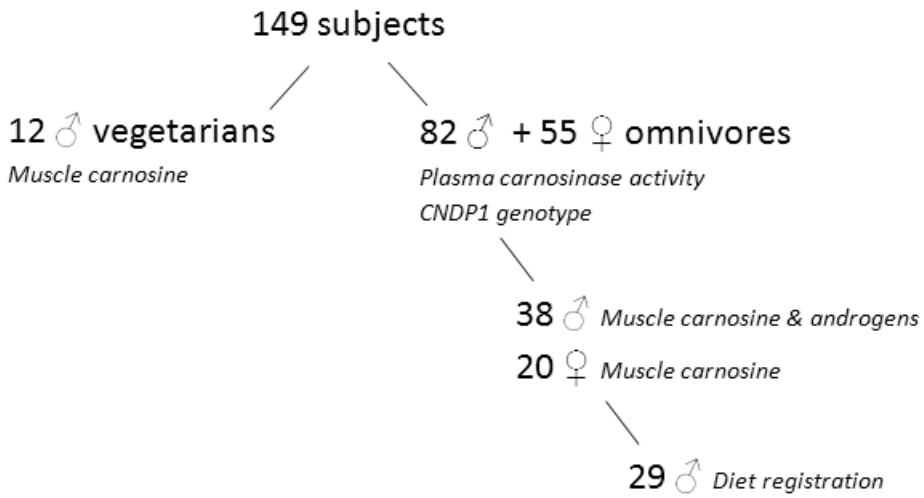

Figure 1. Overview of subject population. Subjects with only plasma are for comparison of *CNDP1* activity between sexes and for relation between *CNDP1* genotype and plasma carnosinase activity

Venous blood sampling. For measurement of circulating androgens, plasma carnosinase activity and for *CNDP1* genotyping, blood of the antecubital vein was withdrawn in heparin tubes. Blood samples were centrifuged and the plasma and blood cells were stored separately at -80°C until further analyses.

Genotyping. A more detailed description of the *CNDP1* genotype determination is explained in the study of Mooyaart *et al.* (29). In brief, a standard PCR protocol was used with primers 5-FAM-GCGGGGAGGGTGAGGAGAAC (forward) and GGTAACAGACCTTCTTGAGGAATT-TGG (reverse). The denaturing, annealing and extension temperatures were 94°C, 60°C and 72°C, respectively. After PCR amplification, fragment analysis was performed on the ABI3130 analyzer (Perkin Elmer) to determine the fragment length corresponding with the different genotypes. Each peak corresponded with the number of leucine repeats on each allele. A 157, 160 and 163 base pair product corresponded with 5, 6 and 7 CTG codons encoding for 5, 6 and 7 leucine repeats, respectively. The 5-5 and the 5-6 *CNDP1* genotypes are widespread and each represent approximately 40% of the population. The 6-6 genotype is present in ± 12% of the population while the 5-7 (± 4%) and 6-7 (± 4%) *CNDP1* genotypes are less common (14; 20; 29).

Plasma carnosinase activity. Heparin plasma samples of 82 men and 55 women were used to quantify the plasma carnosinase activity. Plasma carnosinase activity was

determined according to the method described by Teufel *et al.* (47). Briefly, the reaction was initiated by addition of substrate (L-carnosine) to a plasma sample and stopped after 10 min of incubation at 37°C by adding 1% sulphate salicylic acid. The maximum increase was used for determining the maximum activity. Liberated histidine was derivatized with o-phtaldialdehyde (OPA). Fluorescence was measured by excitation at 360 nm and emission at 460 nm. The intra- and inter-assay variations were respectively 7% and 25%. The lowest carnosinase activity detectable was 0.117 µmol/ml/h.

Androgens. Heparin plasma samples of 38 men were analyzed using a commercial immunoassay kit to determine the plasma concentrations of testosterone (Orion Diagnostica, Espoo, Finland) and LH (ECLIA, Roche Diagnostics). The free fraction of testosterone was calculated from plasma testosterone, SHBG, and albumin concentrations using a previously validated equation (49).

Dietary beta-alanine intake. A subgroup of 29 male omnivore subjects completed a questionnaire about their meat and fish consumption during 2 weeks to quantify daily dietary beta-alanine intake, as described in the study of Baguet *et al.* (3).

Statistics. The *CNDP1* genotypes of exon 2 were categorized based on the leucine repeat length (5-5, 5-6, 5-7, 6-6, 6-7). Independent sample T-tests were used to evaluated the effect of gender, age and *CNDP1* genotype on the muscle carnosine content. An univariate analysis of variance with age as covariate was used to verify the effect of vegetarianism on muscle carnosine content. The correlation between genotype and carnosinase activity was assessed by an univariate analysis of variance with carnosinase activity as dependent and both gender and *CNDP1* genotype as independent variables (post hoc: pairwise comparisons). The other possible determinants were analyzed by bivariate correlations. All statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA) and a p-value < 0.05 was considered to be statistically significant.

Results

Gender. As illustrated in figure 2A, men had a higher muscle carnosine content in comparison to women ($p \leq 0.004$), but the magnitude of this sex-related difference depends on the type of muscle. The soleus and gastrocnemius showed a 36% and 28% larger carnosine content in men, whereas the difference was 82% in tibialis anterior.

The plasma carnosinase activity was significantly higher ($p < 0.001$) in women (7.20 ± 1.62 µmol/ml/h) in comparison with men (5.79 ± 1.58 µmol/ml/h; figure 2B).

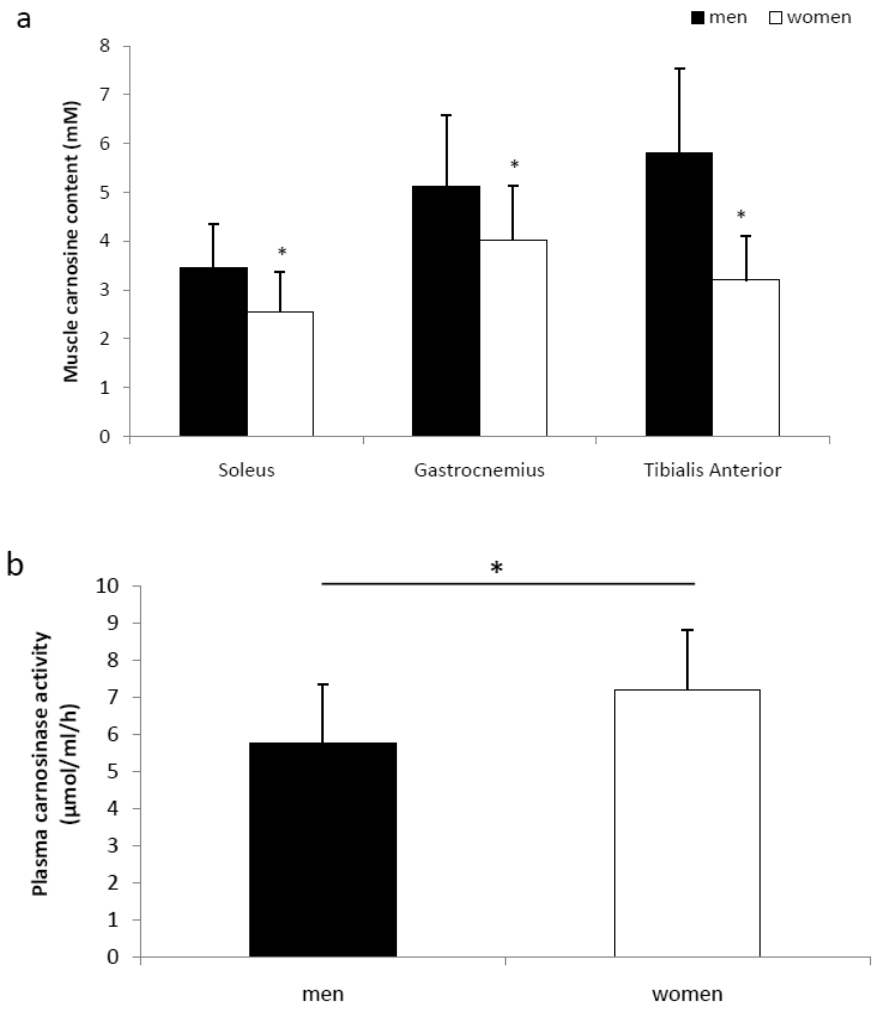

Figure 2. a/ The carnosine content of men (n = 38) versus women (n = 20) in soleus, gastrocnemius and tibialis anterior, measured by proton spectroscopy, * different from men ($p \leq 0.004$). b/ The plasma carnosinase activity of the female (n = 55) is 24,3% higher in comparison with the carnosinase activity of the male population (n = 82) ($p < 0.001$)

Age. The carnosine concentration in the M. soleus (n = 58, p = 0.049; r = -0.260, figure 3) declines with age in the adult range of 19-47 years (M. gastrocnemius: p = 0.112; r = -0.211, M. tibialis anterior: p = 0.482; r = -0.096). In the same group, age did not significantly correlate with plasma carnosinase activity (p = 0.355; r = 0.124).

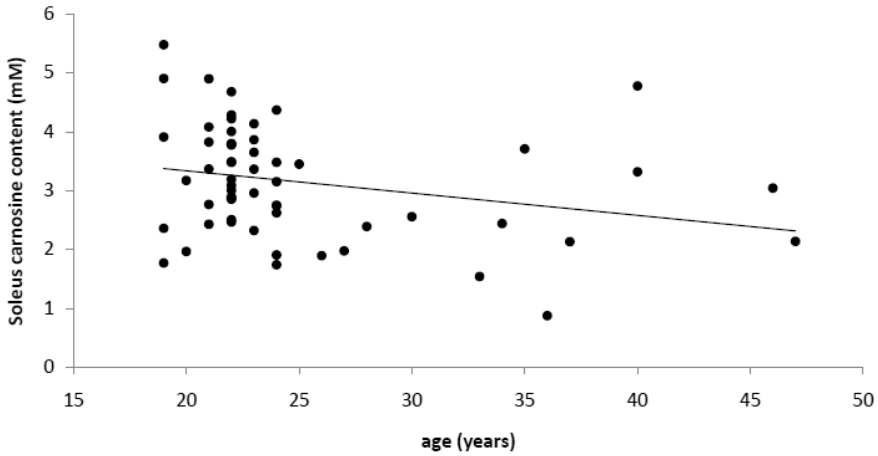

Figure 3. Effect of age on muscle carnosine content in both male and female omnivores. There is a negative correlation of age and carnosine concentration in soleus ($p < 0.05$, $r = -0{,}260$, $n = 58$)

Androgens. In order to elucidate the mechanisms of the age and gender related effects on the muscle carnosine content, we measured plasma testosterone and free testosterone concentrations. The mean (± SD) total testosterone and free testosterone plasma levels in the male reference population were, respectively, 538.6 ± 140.3 $ng \cdot dl^{-1}$ and 11.7 ± 3.5 $ng \cdot dl^{-1}$. Neither of them correlated with muscle carnosine content (soleus, gastrocnemius and tibialis anterior) nor with carnosinase activity ($0.238 \leq p \geq 0.921$). A scatter plot of plasma free testosterone content with M. soleus carnosine content is depicted in figure 4. The plasma total and free testosterone concentration is inversely related to the subjects' age ($p < 0.02$; $r = -0.410, -0.402$; respectively).

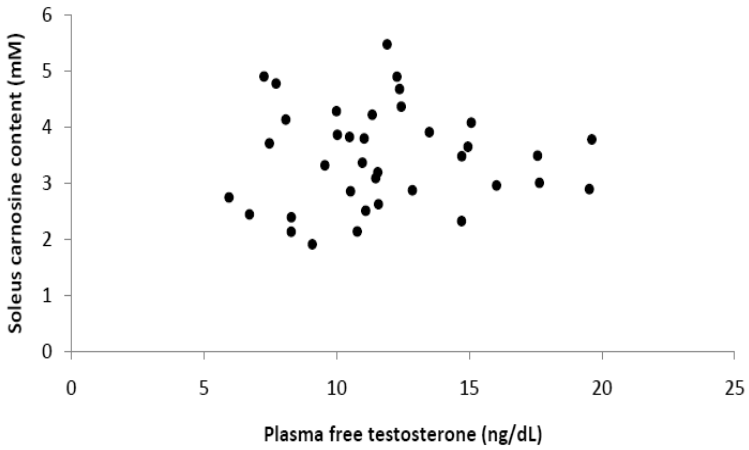

Figure 4. The lack of correlation between plasma free testosterone and soleus carnosine content (p = 0.921). Similar results were obtained for gastrocnemius and tibialis anterior (p = 0.851 and p = 0.794, respectively) and for the correlation of the muscle carnosine content with testosterone, luteinizing hormone (LH) and sex hormone binding globulin (SHBG) (p > 0.05)

Daily beta-alanine intake. Long-term vegetarianism (> 8 years) is associated with declined muscle carnosine stores (figure 5). Vegetarians have lower carnosine levels of 17% in M. soleus (p = 0.05) and 26% in M. gastrocnemius (p = 0.009) and a trend to a lower carnosine content (20%) in M. tibialis anterior (p=0.068) compared to omnivores. However, the significance of the effect of vegetarianism on soleus carnosine content disappeared, when the data were corrected for age (p = 0.304), whereas this was not the case in M. gastrocnemius nor in tibialis anterior. The mean (± SD) daily average beta-alanine ingestion by meat and fish consumption in a subgroup of 29 omnivore male subjects was 0.332 ± 0.144g. Within a normal Western omnivore diet, beta-alanine intake by meat and fish consumption is not a determinant of the muscle carnosine content, as there is no correlation between beta-alanine ingestion and muscle carnosine content ($0.671 \leq p \geq 0.885$) nor a difference in muscle carnosine content between subjects with a low (< 0.332 $g \cdot day^{-1}$) or a high (> 0.332 $g \cdot day^{-1}$) intake of beta-alanine ($0.296 \leq p \geq 0.562$).

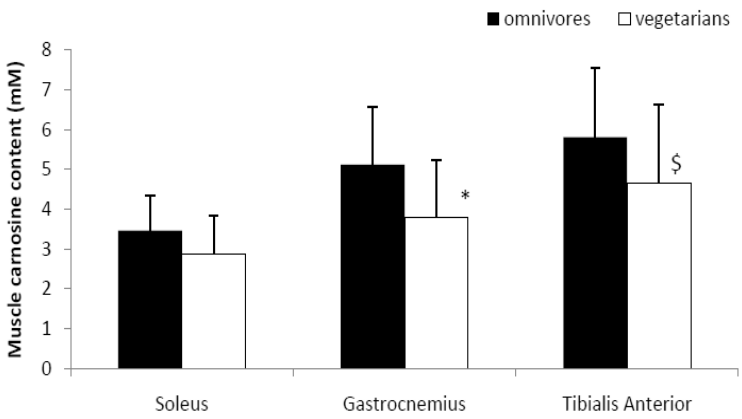

Figure 5. Male vegetarians (n = 12) have a lower muscle carnosine content (* $p < 0.05$ and \$ $p < 0.10$) in comparison with male omnivores (n = 38)

Genotype and plasma carnosinase activity. The most common *CNDP1* genotypes were 5-5 (35.8%) and 5-6 (38%). The 5-7, 6-6 and 6-7 *CNDP1* genotypes were detected in respectively 7.3%, 15.3% and 3.6% of the subjects. Figure 6a shows that the plasma carnosinase activity is dependent on the *CNDP1* genotype ($p = 0.054$). The plasma carnosinase activity of the 5-5 genotype is lower compared to the 5-6 ($p = 0.05$) and to the 6-6 genotype ($p = 0.025$). Also the 6-7 alleles show a lower plasma carnosinase activity than the 6-6 alleles ($p = 0.035$). The relation between the most frequent *CNDP1* genotypes (5-5 and 5-6) and the muscle carnosine content is depicted in figure 6b. The muscle carnosine content of the individuals with the 5-5 *CNDP1* genotype is similar to the carnosine levels of the subjects with the 5-6 *CNDP1* genotype, in both M. soleus, gastrocnemius and tibialis anterior ($0.393 \leq p \leq 0.576$). Likewise, there is no correlation between muscle carnosine levels and the activity of the carnosine degrading enzyme in plasma ($0.154 < p < 0.744$).

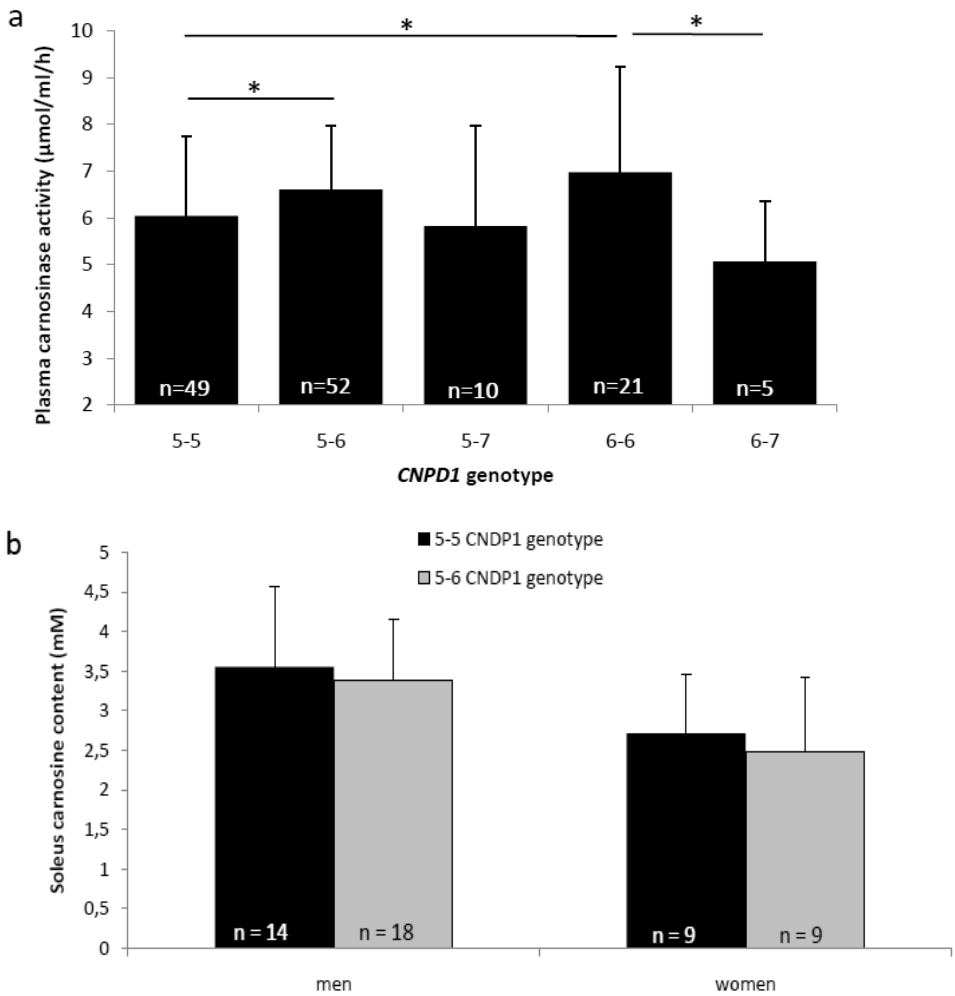

Figure 6. a/ Mean (± SD) plasma carnosinase activity (µmol/ml/h) categorized by amount of leucine repeats in *CNDP1* gene of both 82 males and 55 females. b/ The carnosine content of individuals with the 5-5 *CNDP1* genotype in comparison with individuals with the 5-6 *CNDP1* genotype ($p > 0.05$)

Discussion

Carnosine is synthesized in skeletal muscles from histidine and beta-alanine, with the latter being the rate-limiting precursor. It is demonstrated that an increased availability of beta-alanine, by means of oral supplementation, results in higher muscle carnosine levels (for review see: (8; 37). This is the first peer-reviewed study which shows that a complete and long-lasting restriction of beta-alanine from the diet, as is the case in habitual vegetarians (lacto-ovo or vegan, > 8 yrs), results in lower intramuscular carnosine concentrations as compared with omnivorous subjects. In a proceedings abstract by Harris *et al.* (16), it was shown that female vegetarians have a 45% lower carnosine content in M. vastus lateralis compared with male sports science students. We observed a 26% lower carnosine content in the gastrocnemius of male vegetarians compared to male omnivores. The lower muscle carnosine levels in vegetarians implies that it may be important for vegetarian athletes, involved in high-intensity exercise, to compensate a possible shortage of muscle carnosine by means of beta-alanine supplementation, as also recommended for creatine (13). However, the decreased intramuscular total creatine content in vegetarians versus omnivores is less pronounced, namely 11.1% (7). As there is no difference in muscle carnosine content between individuals with a higher or a lower beta-alanine intake within a normal western omnivore diet, we can conclude that only the supplementation with very high doses of beta-alanine and the complete and prolonged restriction of beta-alanine from the diet does influence the carnosine content.

This study confirms the higher muscle carnosine content in males versus females shown by Mannion *et al.* (26), but in the currently studied muscles of the lower leg, the gender-based differences are even more pronounced (28-82%), compared to the differences in M. quadriceps femoris of the previous study (21%). To the best of our knowledge, no other metabolite in the muscle shows such a large gender-dependent difference. In that light, it is interesting that a sex-specific effect was seen in the relation between diabetic nephropathy and the *CNDP1* genotype (30). Additionally, this gender difference could contribute to the lower high-intensity exercise capacities of women compared to men. Besides the effect of gender on the muscle carnosine content, there is also a negative correlation between age and muscle carnosine content. Stuerenburg *et al.* (42) described a correlation coefficient of -0.4 in patients with neuromuscular diseases ranging from 20 to 80 years. This study is the first that shows this negative

correlation in a healthy adult population (19-47 years). Our cross-sectional data suggest a decline in soleus carnosine content of 1.2%/yr. However, the majority of the subjects are younger than 30 years and the confirmation of these data in an older healthy adult population is recommended. A number of possible factors exist as to why the carnosine levels are affected by gender and age. We hypothesize that both muscle fiber type and circulating androgen levels could be responsible for this gender and age based distinction in muscle carnosine levels.

Despite the conflicting reports regarding the percentage muscle fiber type proportion amongst gender and age, it is generally acknowledged that women and elderly people are characterized with a smaller cross-sectional area of muscle fibers which is most pronounced in fast-twitch fibers (38; 39). Women have 56% smaller cross-sectional area (CSA) of type IIB/X muscle fibers in comparison with men, whereas this gender-induced difference is only 14% in slow-twitch muscle fibers (38; 39). As already mentioned in the introduction, fast twitch muscle fibers are characterized by 30 - 100% higher carnosine levels compared to slow-twitch muscle fibers (15; 18; 23). Thus, it is possible that women and elderly people have lower carnosine levels as a result of a smaller proportion of fast twitch muscle fibers compared to young men.

The hypothesis that circulating androgen levels could affect the muscular carnosine levels is based on the study of Penafiel *et al.* (33) in which they successfully manipulated the carnosine content of murine skeletal muscles by means of subcutaneous testosterone injections in female mice and by removing the testes in male mice. However, we found no correlation between the plasma (free) testosterone levels and muscle carnosine content within a healthy male population. Nevertheless, this does not exclude the possibility that more extreme variations in total or free testosterone, such as overt hypogonadism or exogenous testosterone supplementation do influence muscle carnosine content. This argumentation is supported by the two-fold higher muscle carnosine content of the bodybuilders in the study of Tallon *et al.* (46), in which 5 of the 6 subjects reported to have taken anabolic steroids in the last 6 months.

The observed influence of vegetarianism, gender and age on carnosine content seems to depend on the muscle type under investigation. The gastrocnemius muscle is most affected by dietary beta-alanine restriction, the tibialis anterior is the muscle that displays the largest sexual dimorphism and the age-related effect on muscle carnosine content is only observed in soleus. Muscle-specific differences in expression profiles of e.g. enzymes of carnosine metabolism, amino acid transporters or androgen receptors could be hypothesized.

In line with previous reports, we observed that the *CNDP1* polymorphism affects the plasma carnosinase activity. The higher activity of the carnosine degrading enzyme for the 5-6 and the 6-6 *CNDP1* genotypes compared to the homozygosity for the Mannheim allele (5-5) is confirmed (20; 29). However, it has to be noted that there is a high variation in plasma carnosinase activity within one genotype group (e.g. a range of 2-10 µmol/ml/h in 5-5 genotype). Remarkably, individuals with the 6-7 *CNDP1* genotype have a significantly lower carnosinase activity than individuals with the 6-6 variant. This is in contrast with the suggestion of Janssen *et al.* (20) that both the 6 and 7 leucine alleles can be regarded as gain-of-function mutations associated with a higher enzyme activity and with the results of Riedl *et al.* (36) that the percentage of secreted carnosinase increases with increasing length of the leucine repetitions in the signal peptide. Variations of natural inhibitors of carnosinase such as homocarnosine may account for additional fluctuations in enzyme activity (34). Furthermore, the results of this study reveal that females are characterized with a higher plasma carnosinase activity versus males, confirming and strengthening the non-significant gender-based differences in plasma carnosinase levels which were previously reported (4; 34). Despite the combination of declined muscle carnosine levels and a higher activity of the plasma carnosinase enzyme in both women and elderly people (34), we shown no inverse relationship between both parameters in a gender - and age - mixed population. Similarly, also the polymorphism of the *CNDP1* gene does not determine the muscle carnosine levels. A different compartimentation could underlie this finding, as carnosine is mainly present in the muscle and the enzyme carnosinase is present in the circulation but not (active) in the muscle. Additionally, muscle carnosine could be more sensitive to the carnosine synthetase activity than to the carnosinase activity. However, we are aware that our population is probably too small to completely exclude the possibility that muscle carnosine is related to *CNDP1* genotype. It is therefore necessary to confirm these data in a larger population with a relevant number of subjects with less common genotypes (5-7, 6-6, 7-7).

In conclusion, the results of this study provide evidence for (I) a declined muscle carnosine content in vegetarians which implies that it may be important for vegetarian athletes, involved in high-intensity exercise, to compensate a possible shortage of muscle carnosine by means of beta-alanine supplementation, (II) a marked sexual dimorphism in muscle carnosine levels and plasma carnosinase activity and (III) an independency of the muscle carnosine content to the polymorphism of the *CNDP1* gene and the enzymatic activity of plasma carnosinase.

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The authors declare that they have no conflict of interest.

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8

Summary and general discussion

Diabetic nephropathy is a severe complication of both type 1 and type 2 diabetes. In this thesis, several aspects of diabetic kidney disease relating histopathology, genetics and carnosine metabolism, were investigated.

In **chapter 2** we developed a histopathologic classification scheme for diabetic nephropathy in type 1 and type 2 diabetes. The classification system is based on glomerular lesions, consisting of four classes and reflecting the natural course of diabetic nephropathy. Class I "*Glomerular Basement Membrane Thickening (GBM)*" consists of isolated glomerular basement membrane thickening and only mild, non-specific changes by light microscopy that do not meet the criteria of classes II through IV. Class II "*Mesangial expansion, mild or severe*" is characterized by glomeruli with mild or severe mesangial expansion but without nodular sclerosis (Kimmelstiel-Wilson lesions) or global glomerulosclerosis in more than 50% of glomeruli. A biopsy is classified in class III "*Nodular sclerosis (Kimmelstiel-Wilson lesions)*" if at least one glomerulus with nodular sclerotic lesion (Kimmelstiel-Wilson lesion) is present without changes as described in class IV. Class IV "*Advanced diabetic glomerulosclerosis*" is characterized by more than 50% global glomerulosclerosis with clinical or pathological evidence that the sclerosis is attributable to diabetic nephropathy. We chose a classification scheme based on glomerular lesions because these seem to reflect the natural cause of progressive diabetic nephropathy (1). Furthermore, glomerular lesions are easy to recognize, which is also reflected in the good interobserver agreement of this classification scheme.

Interstitial lesions also contribute to the decline in renal function in diabetic nephropathy, and may be independent factors in the progression of diabetic nephropathy (2). Many studies have shown that the severity of chronic interstitial and glomerular lesions are closely associated (3-6). In our classification scheme a separate evaluation was developed for interstitial and vascular lesions.

A classification system for histopathological lesions in diabetic nephropathy that can be used in both type 1 and type 2 diabetes was proposed, as it is now generally recognized that there is substantial overlap between these two types with respect to histological lesions and renal complications (2;6). Various studies report that the proportion of non-diabetic nephropathies is higher in type 2 diabetic nephropathy (7;8). However, studies which investigated protocol biopsies, did not show such a high proportion of other kidney diseases. Most likely this is due to the fact that in some clinical practices, there is a policy only to perform a renal biopsy to exclude causes of renal disease characterized by proteinuria other than diabetic nephropathy, therefore

selecting for a high proportion of other kidney diseases. The classification system proposed in chapter 2 is meant for diabetic nephropathy only, but it can also serve to classify diabetic nephropathy when it is complicated by a superimposed other disease.

An important question for every histological classification system is whether it is predictive of clinical outcome. The classification scheme proposed in chapter 2 can be used to evaluate protocol biopsies. It would be an interesting undertaking to set up a prospective study, preferably including protocol biopsies of patients with both type 1 and type 2 diabetes, with clearly defined clinical endpoints. Archived renal biopsies with diabetic nephropathy performed for many different clinical indications, at many different time points during the course of the disease and clinical follow-up, were not always readily available. With the increasing demand for classifying the severity of diabetic nephropathy, we advocate a study with protocolized biopsies as previously mentioned. Furthermore, the reproducibility of the classification scheme should be thoroughly investigated, to clarify if it is indeed suitable for clinical practice.

Chapter 3 is focused on the genetic component of diabetic kidney disease. Many genes have been investigated in diabetic nephropathy, but it is unclear which genetic variants are reproducibly associated with diabetic nephropathy. For this study, all genetic variants which were associated with diabetic nephropathy and replicated in an independent dataset were selected from the literature. All subsequent studies of these reproduced variants were sought and were combined in a random-effects meta-analysis. In this study, we found a set of 21 genetic variants which were associated with advanced diabetic nephropathy. Three additional variants were associated in specific subgroups; type of diabetes, stage of diabetic nephropathy and ethnic group. Variants in or near *ACE, AKR1B1* (2 variants)*, APOC1, APOE, EPO, NOS3* (2 variants)*, HSPG2, VEGFA, FRMD3* (2 variants)*, CARS* (2 variants)*, UNC13B, 'CPVL and CHN2',* and *GREM1* (as well as four variants not near to known genes) were associated with diabetic nephropathy and *CCR5* (Asians), *ELMO1* (Asians) and *CNDP1* (type 2 diabetes) in a subgroup. These results support roles for angiotensin converting enzyme, aldose reductase, apolipoproteins C1 and E, oxidative stress with carnosine, nitric oxide and engulfment and cell motility protein 1 as possible key players. Furthermore, it suggests a role for inflammation with a key role for chemokine receptor 5, angiogenesis with a role for vascular endothelial growth factor and erythropoietin, glomerular filtration barrier defects in heparan sulphate proteoglycans, apoptosis through unc-13 homolog B and cell growth and differentiation mediated by gremlin 1 in the pathogenesis of diabetic

nephropathy. Functional studies remain to be performed to establish the precise role of these variants and pathways. Genetic variants initially identified using a genome-wide association approach in and near the *FRMD3*, *CARS*, *ELMO1* and *'CPVL and CHN2'* were detected. Despite their value in locating the vicinity of genomic variants that may be causing diabetic nephropathy, these variants are unlikely to be causative variants themselves (9). A first step in narrowing a genome wide signal to potentially causative variants is to study all the known SNPs which are inherited with the SNP found in the genome wide association scan and investigate if one of these variants has a stronger association with diabetic nephropathy. The functional effect of this variant can then be studied. However, the association can also be explained by rare variants and then extensive sequencing is indicated.

It should also be acknowledged that by selecting only genetic variants that were associated with diabetic nephropathy and where independent replication was available, genetic variants with smaller effect sizes may have been missed. An effect that may have proven significant using pooled analyses. However, by selecting only those genetic variants reproducibly associated with diabetic nephropathy, we reduced the chances of describing false positive associations.

Furthermore, diabetic nephropathy can be prevented or at least delayed by early treatment with angiotensin converting enzyme inhibitors and angiotensin receptor blockers (10). This might result in diabetes patients who are genetically susceptible to diabetic nephropathy but now enter the control group due to successful treatment. This leads to an underestimation of the estimated effect of the genetic variant in relation to diabetic nephropathy.

Chapters 4, 5 and, 6 are focused on one genetic variant associated with diabetic nephropathy, the *CNDP1* gene. Janssen *et al.* (11) found that the 5-5 homozygous genotype of the *CNDP1* gene is associated with a reduced risk of developing diabetic nephropathy. This finding was confirmed in a large study conducted by Freedman *et al.* in European American patients and end-stage diabetic nephropathy (12). The presence of more than 5 leucine repeats has been shown to lead to higher serum carnosinase secretion (13) and more serum carnosinase activity (11). Serum carnosinase degrades carnosine. Carnosine has been shown to delay senescence in cultured human fibroblasts and temporarily reverse the senescence phenotype (14;15). Carnosine is further known to scavenge reactive oxygen species, degrade advanced glycation end products (AGE) (16), inhibit angiotensin converting enzyme (ACE) (17), and reduce the TGF-β induced

synthesis of extracellular matrix components (11). All these properties have protective effects in a diabetic environment.

Chapter 4 reports the 5-5 homozygous *CNDP1* genotype frequency in three ethnic groups in the Netherlands; South Asian Surinamese, African Surinamese and White Dutch. We found that the frequency of the protective genotype for diabetic nephropathy, 5-5 homozygous genotype, was significantly lower in South Asian Surinamese compared to white Dutch and African Surinamese. This finding was confirmed in an independent South Asian Surinamese sample. This low frequency of the 5-5 homozygous genotype found in South Asian Surinamese is likely to be associated with the higher occurrence of diabetic nephropathy in South Asian populations (18-20). This is further supported by the finding that carnosinase activity increases with the amount of leucine repeats among South Asian Surinamese, similar to Caucasians (11). Finally we showed that carnosinase was expressed in kidney, supporting a role for carnosine in the kidney.

Although a direct link between the *CNDP1* gene and diabetic nephropathy still needs to be assessed, this study suggests that the high diabetic nephropathy occurrence in South Asian Surinamese can be partially attributed to the lower frequency of the 5-5 homozygous genotype in this population.

In **chapter 5,** we showed a sex-specific effect of the *CNDP1* genotype in relation to diabetic nephropathy, suggesting that the 5-5 homozygous genotype is only protective in women. The frequency of the 5-5 homozygous genotype was determined in three independent diabetic nephropathy groups. These groups were compared with two control groups; diabetes patients with a low risk of ever developing diabetic nephropathy and a sample from the general population. The former diabetic control group is generally used in the literature, and investigates the relevance of the gene to disease etiology. Compared with this diabetic control group, the 5-5 homozygous genotype frequency was significantly lower in women with diabetic nephropathy in all three cohorts, but not in men. The population control group serves to estimate the genotype risk for the population, showing that women with the 5-5 homozygous genotype have a 2-fold reduced risk of ever developing diabetic nephropathy. Furthermore, this study reports similar frequencies in type 2 diabetes patients and the general population, showing that this association is independent of a genetic susceptibility for type 2 diabetes. Although the mechanism behind the sex-specificity still needs to be elucidated, this study indicates that the association between the *CNDP1* gene and diabetic nephropathy is gender specific.

Carnosine is protective against oxidative stress and hemodynamic damage and this is not confined to diabetic nephropathy. In **chapter 6** the relation between other glomerular diseases and the *CNDP1* gene was investigated. Our results suggest that a higher number of leucine repeats in the *CNDP1* genotype is associated with a faster progression to end stage renal disease in patients with reduced kidney function arising from chronic glomerular inflammatory renal diseases and in patients with renal vascular disease. In line with these results, the mortality risk was increased in chronic glomerulonephritis patients with a higher repeat number, which was also associated with higher serum carnosinase levels. Our data show a correlation between the leucine repeat distribution of the *CNDP1* gene and renal vascular disease; however, there was no relationship with survival. As predicted, patients who developed end stage renal disease because of either polycystic kidney disease or tubulointerstitial nephritis had a *CNDP1* genotype distribution similar to that of healthy controls. These findings support the hypothesis that the high leucine repeat number in *CNDP1* may contribute to microvascular damage. Although this finding has to be replicated in an independent cohort, this study suggests that there is also a role for carnosine in other glomerular diseases.

In **chapter 7** we investigated determinants of muscular carnosine levels. As high carnosine levels are thought to be advantageous, it is important to understand the underlying factors. We studied the relation between muscular carnosine levels and serum carnosinase activity, *CNDP1* genotype, age, vegetarian diet and muscle fiber type. Serum carnosinase activity did reduce the carnosine content, but possibly due to the small number of investigated subjects no relation was shown with the *CNDP1* genotype. Carnosine content was found to decline with age, which could not be explained by the age-related increase in carnosinase activity. Meat-restriction for 8 weeks had no effect on carnosine levels, but in (long-term) vegetarians lower muscular carnosine levels were observed. There was no linear relationship with muscular fiber type and carnosine levels.

In addition, the relation with gender in the carnosine metabolism was studied. Women showed higher carnosinase activity than men and men had higher carnosine levels. This is in line with the idea that from an evolutionary point of view carnosine levels are more beneficial to men. This can be explained by the greater muscular activity needed for males to hunt for food, since carnosine is a buffer for the substances derived from lactic acid that are produced under anaerobic conditions. However, no relation between testosterone and carnosine was found in this study.

Future perspectives

The relation between diabetic nephropathy due to type 2 diabetes and the leucine repeat in exon 2 of the *CNDP1* gene seems to be established. Interesting would be to speculate on what this finding can eventually mean for the diabetic nephropathy patient. The possible relevance of the *CNDP1* genotype in diabetic nephropathy can be roughly divided in two groups; genotypic screening for diabetes patients to be able to predict the risk of developing diabetic nephropathy and novel biological insight in the etiology and pathogenesis of diabetic nephropathy, potentially leading to new therapies (21).

For screening of individuals with an increased risk of disease, the measurement of area under the curve (AUC) of the receiver operating characteristic curve (ROC) is often used. An AUC of 1 is perfect prediction and an AUC of 0.5 is similar to tossing a coin, and therefore not predictive. It has been suggested that an AUC above 0.75 can be considered of predictive value. For pre-symptomatic diagnosis of the general population the AUC should be > 0.99 (22). The *CNDP1* genotype could be used in a genotypic risk score, together with other associated variants related to diabetic nephropathy due to type 2 diabetes. In diabetic nephropathy no such genotypic scores have been tested in contrast to screening for type 2 diabetes. In type 2 diabetes, 18 genetic variants have been established and these genetic variants when combined have shown to reach an AUC of 0.6 in predicting type 2 diabetes onset. This is low when compared to traditional risk factors as only BMI, age and gender which have shown to have an AUC of 0.78 and risk scores, such as the QDS score (a scoring algorithm based on traditional risk factors without the need for laboratory tests) reaches an AUC of 0.85 in predicting type 2 diabetes onset (23). In a different study, the addition of genetic risk factors to clinical risk showed only a slight increase of the AUC (from 0.74 to 0.75), which seems to increase with duration of follow up (24). This indicates that genetic personalized medicine in type 2 diabetes will not easily be achievable in the near future. However, in diabetic nephropathy this still needs to be investigated.

Novel biological insight can, in the most fortunate case, lead to new therapeutic targets. The association between diabetic nephropathy and *CNDP1* (carnosinase) gene, would suggest a role for this enzyme and its substrate (carnosine) in diabetic nephropathy. It was shown that the genotype leading to the lowest carnosinase activity, leaving more carnosine, was associated with a reduced risk of diabetic nephropathy.

This suggests that a possible new therapeutic target for diabetic nephropathy would be increasing carnosine level in diabetes patients. This could be done by supplementing carnosine, reducing the degradation of carnosine by inhibiting carnosinase, or increasing carnosine production by carnosine synthase.

Supplementing carnosine would be an interesting treatment for patients with diabetic nephropathy. Carnosine has been known for its many protective capacities in a diabetic environment and is well tolerated. Carnosine and related compounds have already been used as therapies for cataract (25;26) and gastric ulcers (27). In diabetic mice treated with angiotensin converting enzyme inhibitors, adding carnosine to the diet led to a significant reduction in proteinuria compared to diabetic mice treated with only angiotensin converting enzyme inhibitors (28). In humans, carnosine seems to be hydrolyzed within 2 hours after carnosine is absorbed by the intestine and enters the bloodstream (29). However, when Parkinson patients were treated with carnosine in addition to dopamine (L-dopa), they had a decreased level of protein carbonyls in their blood plasma and increased levels of red cell superoxide dismutase compared to patients treated with only dopamine (L-dopa) (30). This suggests that supplementation with carnosine has a biochemical effect after oral intake even though carnosine is hydrolyzed quickly in serum. This means that carnosine supplementation could theoretically also be of therapeutic value in diabetic nephropathy.

An inhibitor of carnosinase activity is homocarnosine (31). In the literature, no studies have been performed in which patients were treated with homocarnosine. Although we would expect that homocarnosine treatment could reduce the serum degradation of carnosine, high concentrations of homocarnosine might lead to high concentrations of its degradation products gamma-aminobutyric acid (GABA) and histidine. Histidine can be easily converted into histamine and the impact on autonomic nerve activity would be unpredictable. It might result in further progression of renal insufficiency, since progressive renal disease has been shown to be associated with increased nerve activity (31). Future studies could also be focused on the regulation of the carnosinase gene, finding substances which downregulate the expression of *CNDP1*.

Recently the gene coding for carnosine synthase has been identified as a ATP-grasp domain containing protein 1 (*ATPGD1*) (32). The identification of this gene is an essential step in the understanding of carnosine production. It would for example be interesting to investigate in which organs this gene is expressed, and especially if it is expressed in the kidney; if so, in which cells of the kidney. Furthermore, the creation

of knockout mice or mice in which carnosine synthase is overexpressed, could help define the role of carnosine as a buffer, antioxidant, antiglycator and neurotransmitter, of which evidence now mostly comes from *in vitro* studies. It would be interesting to investigate whether overexpressing carnosine synthase in a diabetic kidney disease model, would lead to a milder phenotype of diabetic nephropathy. Finally, it would be interesting to investigate which substances upregulate carnosine synthase, leading to higher carnosine production and a possible therapeutic potential.

Most simple and safe would be to investigate carnosine treatment in diabetic nephropathy, before considering other substances. However, before starting treatment with carnosine, it might be advisable to first understand more about the mechanisms of carnosine metabolism. In chapter 5 of this thesis, a sex-specific effect of the relation between diabetic nephropathy and the *CNDP1* genotype was found. It would be interesting and important to understand the mechanism behind this sex-specific effect. A sex-specific effect has long been described in renal disease (33). In non diabetic progressive chronic kidney disease, female sex is thought to be protective. However, this advantage seems to be lost in diabetic nephropathy. The protective effect in non diabetic renal disease has been ascribed to the effect of estrogens (34). Female diabetes patients seem to have lower estrogens levels (35) and that may explain the loss of female protection in diabetic nephropathy. However, the relationship between sex and diabetic nephropathy seems to be more complicated. Several risk factors have been described to be involved in men while others are present in women (36-38). Of these risk factors, several genetic risk factors (37;39;40) have been documented to be either only involved in women or in men, but these findings have not yet been replicated. In this study, we detected an association in only women and could independently replicate this finding, providing evidence for a sex-specific component in the genetics of diabetic kidney disease.

With an increasing number of leucine repeats (6 or 7 instead of 5) in exon 2 of the *CNDP1* gene, carnosinase activity increases (11). The 5-5 homozygous genotype leads to lower carnosinase activity than that seen in the other genotypes (11), leaving more carnosine free to protect the kidney from oxidative stress. Since men have higher carnosine levels in their muscle tissue and women have slightly higher serum carnosinase levels (41), differences in carnosinase activity due to the different *CNDP1* polymorphisms may have a stronger impact in women.

Another explanation for the sex-specific effect found in this study is that the association between the *CNDP1* gene and diabetic nephropathy is lost in men due to selective survival by cardiovascular disease. As carnosine has shown to be protective against oxidative stress and hemodynamic damage (11;16;42), this might also explain its role in cardiovascular death in diabetic nephropathy patients. Men with diabetic nephropathy due to type 2 diabetes have a higher risk for cardiovascular disease than female diabetic nephropathy patients due to type 2 diabetes (43), therefore this might be more prominent in men. In this thesis we found that men with a diabetes duration < 10 years and the 5-5 homozygous genotype have a significantly lower mortality risk due to cardiovascular disease than patients with more than 10 leucine repeats in the *CNDP1* genotype. We found no difference in cardiovascular death between the different genotypes in women. This finding needs to be replicated, before drawing definite conclusions.

The two hypotheses are clearly different. According to the first hypothesis, carnosine seems to be mainly beneficial in women and suggests that future research should only focus on investigating carnosine metabolism in female diabetic nephropathy patients. The second hypothesis suggests that carnosine might also be protective in macrovascular complications of diabetes and that the sex-specific effect is in fact due to different survival between the sexes. Therefore, it would be helpful to understand the mechanism behind this sex-specific effect for future therapeutic studies.

In summary, the role for the *CNDP1* genotype in the prediction for diabetic nephropathy due to type 2 diabetes is probably limited. On the other hand, the potential new additional treatment for type 2 diabetes patients with carnosine to either prevent or slow down the progression of diabetic nephropathy should be investigated. Furthermore, it is essential to understand the mechanism behind the sex-specific effect of the association between the *CNDP1* genotype and diabetic nephropathy.

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9

Nederlandse samenvatting

Diabetes mellitus, ook wel suikerziekte genoemd, is een ziekte waarbij de patiënt een tekort of relatief tekort heeft aan het hormoon insuline. Insuline zorgt ervoor dat suiker vanuit het bloed wordt opgenomen in het lichaam. Het woord diabetes mellitus is afgeleid van zowel Grieks als Latijn, 'dia' (=διαα) betekent 'door' en bainein (=βαινειν) betekent 'stromen/gaan'. Het woord mellitus komt uit het Latijn en betekent honingzoet. Diabetes mellitus betekent dus 'honing zoete doorstroom', wat duidt op het verhoogde suikergehalte in bloed en urine.

Er zijn verschillende vormen van diabetes mellitus, waarbij de meest voorkomende type 1 diabetes mellitus (ook wel jeugddiabetes of insuline afhankelijke diabetes genoemd) en type 2 diabetes mellitus (ook wel ouderdomsdiabetes of insuline onafhankelijke diabetes genoemd) zijn. Bij type 1 diabetes mellitus worden de insuline-producerende cellen in de alvleesklier door het lichaam afgebroken en is de patiënt levenslang afhankelijk van insuline toediening. Bij type 2 diabetes mellitus is sprake van een combinatie van insuline resistentie, een fysiologische conditie waarbij insuline minder effectief wordt in het verlagen van het bloedsuikergehalte, en verminderde insuline aanmaak. Type 2 diabetes mellitus is geassocieerd met overgewicht en afvallen is dan ook de beste therapie. Verder zijn er middelen om de insuline resistentie of de suiker opname te verlagen. Mocht dit niet voldoende werken, dan worden deze patiënten ook met insuline behandeld.

Alle vormen van therapie zijn er op gericht om hoge bloedsuikerspiegels te normaliseren. Als de suikerspiegels in het bloed langdurig te hoog zijn, leidt dit tot schade aan de bloedvaten. Deze schade is de oorzaak van latere complicaties die kunnen optreden bij diabetes mellitus. De complicaties zijn grofweg in te delen in aantasting van de kleine vaten (diabetische nier- en oogziekte en diabetische voet), grote vaten (hartziekte) en zenuwen (verminderd gevoel in handen en voeten, verminderde maaglediging). Dit promotieonderzoek richt zich op diabetische nierziekte.

Diabetische nierziekte is de belangrijkste oorzaak van terminaal nierfalen, waarbij een transplantatie of dialyse noodzakelijk is. Diabetische nierziekte begint met een compensatoire hyperfiltratie van de zeeflichaampjes van de nier, de glomeruli, die het bloed filteren. Onder de microscoop is dan een verdikking van de basaalmembraan van glomeruli te zien. Later treedt eiwitverlies in de urine op. Naar mate de ziekte vordert zal aanzienlijk eiwitverlies optreden, ook wel proteïnurie genoemd. Als laatste neemt de functie van de nier af tot het punt waarop deze onvoldoende functioneert en dialyse of transplantatie noodzakelijk is. Histologisch treedt na verdikking van de

basaalmembraan toename van het mesangium op. Het mesangium is onderdeel van de glomerulus en zorgt voor onder andere de stevigheid en beweeglijkheid van het vaatbed in de glomerulus. In een later stadium treden ook de zogenaamde Kimmelstiel-Wilson laesies op. Dit zijn nodulaire ophopingen waarvan men nog niet precies de ontstaanswijze weet. Als laatste treedt verbindweefseling van de hele glomerulus op, zogenaamde glomerulosclerose.

In **hoofdstuk 2** wordt een histopathologische classificatie van diabetische nierziekte voorgesteld, gebaseerd op het bovengenoemde ziektebeloop. Klasse I is gedefinieerd als basaalmembraan verdikking van de glomeruli en een verder normale histologie. Een biopt wordt in klasse IIa of b geclassificeerd bij respectievelijk milde of ernstige mesangiale toename. Een biopt wordt als klasse III gescoord als het minimaal één overtuigende Kimmelstiel-Wilson laesie bevat. Klasse IV bevat het laatste stadium met glomerulosclerose in meer dan 50% van de glomeruli in het biopt. Deze classificatie is opgesteld door een internationale groep van experts en is de eerste pathologische classificatie voor diabetische nierziekte die bruikbaar is voor klinische doeleinden. Deze classificatie geldt voor zowel diabetische nierziekte door type 1 als door type 2 diabetes mellitus. De classificatie zal nog moeten worden getest op reproduceerbaarheid en klinische relevantie.

Als diabetische nierziekte optreedt, gebeurt dat meestal ongeveer 10 jaar na het ontstaan van diabetes mellitus. Sommige patiënten zijn gevoeliger voor deze complicatie dan anderen en het blijkt dat patiënten die 15 jaar na het ontstaan van diabetes nog geen diabetische nierziekte hebben ontwikkeld, een grote kans hebben om dit later ook niet meer te ontwikkelen. Dit, tesamen met de bevinding dat diabetische nierziekte clustert in families en de prevalentie van diabetische nierziekte verschilt tussen etnische groepen, heeft tot het idee geleid dat een genetische predispositie bestaat voor het ontwikkelen van diabetische nierziekte.

In het werk beschreven in **hoofdstuk 3** van dit proefschrift werd de medische literatuur onderzocht op zoek naar genetische varianten geassocieerd met diabetische nierziekte. Er zijn veel onderzoeken gepubliceerd over genetische varianten die mogelijk geassocieerd zijn met diabetische nierziekte. Een groot deel hiervan kon tot dusver niet worden gereproduceerd en berust waarschijnlijk op toeval. Zodoende werden in deze meta-analyse alleen de genetische varianten meegenomen waarvoor tenminste in twee onafhankelijke onderzoeken een associatie werd gevonden met diabetische nierziekte. Op het moment dat een genetische variant meermalen geassocieerd was

met diabetische nierziekte werden alle studies met betrekking tot deze genetische variant verzameld en samengenomen in de meta-analyse. Een meta-analyse is een statistische methode om resultaten van verschillende studies te kunnen samennemen. De genetische varianten die in de meta-analyse significant geassocieerd waren met diabetische nierziekte waren gelegen in of dichtbij de volgende genen: *ACE, AKR1B1* (2 varianten)*, APOC1, APOE, EPO, NOS3* (2 varianten)*, HSPG2, VEGFA, FRMD3* (2 varianten)*, CARS* (2 varianten)*, UNC13B, CPVL/CHN2,* en *GREM1*. Verder waren er nog genetische varianten geassocieerd met diabetische nierziekte in alleen type 2 diabeten en Aziaten. Het betreft genetische varianten in de volgende genen: *CCR5* en *ELMO1* bij Aziaten en *CNDP1* in type 2 diabetes mellitus. Verder onderzoek naar het werkingsmechanisme van deze genen in relatie tot diabetische nierziekte kan leiden tot nieuwe inzichten in de ontstaanswijze en het ziektebeloop van diabetische nierziekte en nieuwe behandelingsmogelijkheden.

Hoofdstukken 4, 5 en 6 gaan over één van de genen die in hoofdstuk 3 beschreven zijn, namelijk het *CNDP1* gen. *CNDP1* codeert voor het enzym carnosinase. Dit is een enzym dat de stof carnosine afbreekt. Carnosine is een dipeptide, met verscheidene beschermende effecten. Het is een antioxidant, maar kan ook werken als een buffer, als een potentiële bloeddrukverlager. Daarnaast helpt het mesangiale matrix-eiwitten expansie voorkomen en het kan producten die ontstaan door te hoog suiker in het bloed helpen afbreken (de zogenaamde advanced glycation end products). Er is een genotype van dit gen, de 5-5 homozygoot, dat leidt tot een verminderde carnosinase secretie uit de cel, hetgeen weer leidt tot verminderde carnosinase activiteit in het bloed. Hierdoor wordt er minder carnosine afgebroken en blijft er meer carnosine over om met name de nier te beschermen tegen complicaties. Uit eerdere studies is gebleken dat type 2 diabetes mellitus patiënten met het 5-5 homozygoot genotype een 2 maal verlaagde kans hebben op het ontwikkelen van diabetische nierziekte. Bij type 1 diabetes mellitus is deze relatie niet gevonden.

In het werk beschreven in **hoofdstuk 4** werd de frequentie van het 5-5 homozygoot *CNDP1* genotype in meerdere etnische groepen onderzocht: Creoolse Surinamers, Hindoestaanse Surinamers en blanke Nederlanders. De Hindoestaanse Surinamers hebben vaker diabetes mellitus en diabetische nierziekte. Het is dan ook met name deze populatie die een lagere frequentie had van het beschermende genotype, de 5-5 homozygoot. Deze bevinding konden wij reproduceren in een onafhankelijke groep van Hindoestaanse Surinamers. Verder is gekeken naar de relatie tussen het *CNDP1*

genotype en de carnosinase activiteit in de Hindoestaanse Surinamers. Net als bij blanke Nederlanders, hebben Hindoestaanse Surinamers met het 5-5 homozygoot genotype een lagere carnosinase activiteit. De lagere frequentie van het beschermende 5-5 homozygoot genotype bij Hindoestaanse Surinamers zou één van de verklaringen kunnen zijn waarom deze populatie een verhoogd risico heeft op het ontwikkelen van diabetische nierziekte.

In het werk beschreven in **hoofdstuk 5** werd onderzocht of de relatie tussen diabetische nierziekte en het *CNDP1* gen verschilt tussen mannen en vrouwen. In deze studie zijn mannen en vrouwen met diabetische nierziekte afzonderlijk vergeleken met respectievelijk mannen en vrouwen met diabetes mellitus gedurende meer dan 15 jaar zonder diabetische nierziekte. Aangezien uit eerdere studies is gebleken dat er alleen een relatie bestaat met type 2 diabetes mellitus, beperkt deze studie zich tot type 2 diabetes mellitus patiënten. Het beschermende effect van het 5-5 homozygoot genotype bleek alleen aanwezig bij vrouwen en niet bij mannen. Dit werd in 3 onafhankelijke groepen gevonden. Verder werden deze 3 groepen met diabetische nierziekte vergeleken met een controlegroep uit de populatie, om een inschatting te kunnen maken over wat de kans is dat iemand met het 5-5 homozygoot genotype diabetische nierziekte ontwikkelt. Vrouwen met het 5-5 homozygoot genotype hebben een 2 keer kleinere kans op het ontwikkelen van diabetische nierziekte, terwijl bij mannen geen relatie met diabetische nierziekte werd gevonden. Deze studie laat zien dat alleen bij vrouwen een relatie tussen het *CNDP1* gen en diabetische nierziekte bestaat, maar de precieze reden voor deze bevinding moet nog nader worden onderzocht.

Aangezien carnosine een antioxidant is en verder veel andere beschermende eigenschappen heeft, zou het goed kunnen dat het 5-5 homozygoot genotype ook beschermend werkt bij andere nierziektes. Uit het onderzoek beschreven in **hoofdstuk 6** is gebleken, dat bij glomerulaire nierziekte (net als diabetische nierziekte) een relatie bestaat met het *CNDP1* gen. Ook werd een relatie met mortaliteit bij dialysepatiënten en het *CNDP1* gen in glomerulonefritis-patiënten gezien.

In het onderzoek beschreven in **hoofdstuk 7** werd gekeken naar welke determinanten de carnosine concentratie in de spier beïnvloeden. Carnosine is in hoge concentraties aanwezig in de spier. Dit onderzoek is uitgevoerd in samenwerking met sportwetenschappers in België en heeft een tweeledig doel. Enerzijds om te kijken welke stoffen carnosine beïnvloeden voor eventuele verbetering van sportprestaties. Anderzijds is deze studie opgezet om meer inzicht te krijgen in het carnosine-metabolisme.

Mogelijk kan dit inzicht ook leiden tot nieuwe behandelingsmogelijkheden om carnosine spiegels te verhogen ter bescherming van diabetische nierziekte. Verschillende factoren die de carnosine-concentratie zouden kunnen beïnvloeden werden onderzocht, waaronder carnosinase activiteit in het bloed, *CNDP1* genotype, leeftijd, vegetarisch dieet en spiervezel type. Er was een verwachte relatie tussen carnosinase activiteit en hoeveelheid carnosine, maar niet met het *CNDP1* genotype. Carnosine in de spier nam af met de leeftijd, wat niet kon worden verklaard door de leeftijd-gerelateerde toename van carnosinase. Een vegetarisch dieet gedurende 8 weken had geen effect op de hoeveelheid carnosine in de spier. Langdurig vegetarisme heeft echter wel een effect op de hoeveelheid carnosine in de spier. Er werd geen relatie gevonden tussen het spiervezel type en carnosine spiegels. Verder is het effect van het geslacht op het carnosine metabolisme onderzocht. Vrouwen hebben een hogere carnosinase activiteit dan mannen en mannen een hogere carnosine concentratie in de spier. Deze bevinding zou de basis kunnen vormen voor het in hoofdstuk 5 gevonden geslachtsspecifieke effect. Aangezien vrouwen een hogere carnosinase activiteit hebben en relatief weinig carnosine, hebben vooral zij baat bij het hebben van een 5-5 homozygoot genotype. De mogelijke toename van carnosine spiegels onder invloed van testosteron kon in onze studie niet worden aangetoond.

Samenvattend heeft de inhoud van dit proefschrift nieuw licht geworpen op de pathologie, genetica en het carnosine metabolisme met betrekking tot diabetische nierziekte.

A

Online supplements

Contents

Chapter 2 – Figure 1. Scoring form for glomerular lesions, extraglomerular lesions and other features.

Chapter 3 – ESM Table 2 - Details of the articles which were included in this meta-analysis study
(ESM Table 1 and ESM figure 1-36 can be found online on the Diabetologia website)

Chapter 5 – Hardy-Weinberg equilibrium, Sensitivity analysis, Permutation

Figure 1. Scoring form for glomerular lesions, extraglomerular lesions and other features.

RPS DN Working Group score sheet

Case Number	
Pathologist	
Date	
Stainings evaluated	

EM Avg GBM size (nm) ☐

A. Glomerular lesions	*Tally mark*	*no.*
Total # of evaluated glomeruli		
Normal glomeruli		
Global glomerulosclerosis		
Nodular lesions		
Mesangial expansion		
Mild		
Severe		

Name	#	*Criteria*	**Classify into glomerular class:** ?
EM proven DN	I	GBM > 395 nM (female), > 430 (male)	
Mild Mesangial Expansion	II A	Mild mesangial expansion in >25%	
Severe Mesangial Expansion	II B	Severe mesangial expansion in >25%	
Nodular Sclerosis	III	At least one convincing nodular lesion	
Glomerosclerosis with signs	IV	Global glomerular sclerosis in >50% of	
of DN		glomeruli in proven DN	

EM, electronmicroscopy; TA, tubular atrophy; IF, interstitial fibrosis

B. Extraglomerular Lesions		*Circle score*
Tubular atrophy (%)	< 25 % tubular atrophy	0
	25-50 % tubular atrophy	1
	>50 % tubular atrophy	2
Interstitial fibrosis (%)	< 25 % interstitial fibrosis	0
	25-50 % interstitial fibrosis	1
	>50 % interstitial fibrosis	2
Interstitial inflammation		
	absent	0
	Infiltration only in relation to IF or TA	1
	Infiltration in areas without IF or TA	2
Large vessels present?		Y / N
Arteriosclerosis (score worst artery)		
	No intimal thickening	0
	intima thickened and < thickness of media	1
	intima thickened and > thickness of media	2
Arteriolar hyalinosis	absent	0
	at least one case of arteriolar hyalinosis	1
	>1 case of arteriolar hyalinosis	2

C. Other features

Capsular drop present / absent

Fibrin cap present / absent

Chapter 3

Electronic supplementary material

ESM Table 2 Details of the articles which were included in this meta-analysis study

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
ACE											
rs179975	Ahluwalia et al., 2009 (1)	EDN[b]+ADN[c]	Normoalb[d] ≥10 years of diabetes	240			255	Case–control	India	2	1.57 (1.21– 2.04)
	Araz et al., 2001 (2)	EDN	Normoalb	62			123	Case–control	Turkey	2	1.00 (0.64–1.54)
	Arfa et al., 2008 (3)	EDN	Normoalb ≥10 years of diabetes	54			51	Case–control	Tunisia	2	1.24 (0.70–2.21)
	Canani et al., 2005 (4)	EDN+ADN	Normoalb	203			609	Case–control	Brazil	2	1.22 (0.97–1.53)
	Chowdhury et al., 1996 (5)	EDN	Normo-/microalb[e] >15 years of diabetes (no proteinuria)	242			166	Case–control	UK	1	1.04 (0.78–1.38)
	Demurov et al., 1997 (6)	EDN	Normoalb (diabetes without complications)	56			76	Case–control	Russia	1	1.98 (1.19–3.30)
	Doi et al., 1996 (7)	EDN+ADN	Normoalb >10 years of diabetes	100			124	Case–control	Japan	2	1.55 (1.05–2.28)
	Fradin et al., 2002 (8)	EDN	Normoalb	39			118	Case–control	France	2	0.86 (0.51–1.44)
	Gohda et al., 2001 (9)	EDN+ADN	Normoalb >15 years of diabetes	416	289	127	212	Case–control	Japan	2	1.09 (0.86–1.39)
	Grzeszczak et al., 1998 (10)	EDN+ADN	Normoalb ≥10 years of diabetes	127			254	Case–control	Poland	2	0.91 (0.67–1.23)
	Guitterez et al., 1997 (11)	EDN	Normoalb	20			100	Case–control	Spain	2	1.27 (0.62–2.62)
	Ha et al., 2003 (12)	ADN	Stable kidney function >15 years	140			99	Follow-up	Korea	2	1.84 (1.27–2.66)
	Hadjadj et al., 2001 (13)	EDN+ADN	Normoalb ≥3 years of diabetes	24	18	6	251	Case–control	France	1	0.84 (0.46–1.52)
	Hadjadj et al., 2007 (14)	EDN+ADN	Normoalb ≥15 years of diabetes (without anti-hypertensiva)	380			382	Case–control	Denmark	1	1.04 (0.85–1.27)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
		EDN+ADN	Normoalb ≥15 years of diabetes (without anti-hypertensiva)	385			468	Case–control	Finland	1	1.09 (0.90–1.32)
		EDN+ADN	Normoalb ≥15 years of diabetes (without anti-hypertensiva)	277			273	Case–control	France	1	1.45 (1.14–1.84)
	Hibberd et al., 1997 (15)	EDN+Ret[f]	Normoalb ≥20 years of diabetes	72			86	Case–control	UK	1	0.69 (0.44–1.10)
	Kimura et al., 1998 (16)	EDN+ADN+Ret	Normo-/microalb >15 years of diabetes	98			110	Case–control	Japan	2	0.96 (0.65–1.43)
	Marre et al., 1997 (17)	EDN+ADN	Normoalb	233	126	107	157	Case–control	France	1	1.41 (1.06–1.88)
	Miura et al., 1999 (18)	EDN	Normoalb >10 years of diabetes	32			103	Case–control	Japan	1	0.98 (0.55–1.76)
	Mollsten et al., 2008 (19)	EDN+ADN	Normoalb ≥20 years of diabetes (without anti-hypertensiva)	48			197	Case–control	Sweden	1	0.81 (0.52–1.27)
	Nakajima et al., 1996 (20)	EDN+Ret	Normoalb	54			41	Case–control	Japan	2	1.25 (0.68–2.28)
	Naresh et al., 2009 (21)	EDN+Ret	No nephropathy	30			30	Case–control	India	2	3.02 (1.43–6.38)
	Ng et al., 2006 (22)	EDN+ADN	Normoalb; 6 years of diabetes	291			167	Case–control	USA	2	1.10 (0.84–1.45)
	Nikzamir et al., 2009 (23)	EDN	Normoalb	48			145	Case–control	Iran	2	4.33 (2.54–7.41)
	Ohno et al., 1996 (24)	EDN	Normoalb	25			53	Case–control	Japan	2	2.55 (1.24–5.21)
	Ortega-Pierres et al., 2007 (25)	EDN	Normoalb	45			116	Case–control	Mexico	2	3.20 (1.89–5.43)
	Park et al., 2005 (26)	ADN	Normoalb >15 years of diabetes	103			88	Case–control	Korea	2	1.71 (1.13–2.57)
	Prasad et al., 2006 (27)	ADN+Ret	Normoalb ≥10 years of diabetes	196			225	Case–control	India	2	1.36 (1.03–1.79)
	Shestakova et al., 2006 (28)	EDN	Normoalb >20 years of diabetes	63			66	Case–control	Russia	1	1.36 (0.83–2.22)
	Shin Shin et al., 2004 (29)	EDN	Normoalb	82			50	Case–control	Korea	2	0.68 (0.41–1.11)
	So et al., 2006 (30)	EDN	Normoalb	421			1225	Case–control	Hong Kong	2	0.97 (0.82–1.14)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
	Taniwaki et al., 2001 (31)	EDN+ADN	Normoalb	42	22	20	69	Case–control	Japan	2	1.03 (0.59–1.81)
	Tarnow et al., 1995 (32)	EDN+Ret	Normoalb	198			190	Case–control	Denmark	1	1.01 (0.76–1.34)
	Thomas et al., 2001 (33)	EDN	Normoalb	51			255	Case–control	China (Han)	2	0.88 (0.55–1.40)
	Tomino et al., 1999 (34)	EDN	Normoalb >10 years of diabetes	414			407	Case–control	Japan	2	0.96 (0.79–1.18)
	van Ittersum et al., 2000 (35)	EDN	Normoalb	30			188	Case–control	Netherlands	1	0.85 (0.49–1.48)
	Viswanathan et al., 2001 (36)	EDN	Normoalb+no Ret	86			23	Case–control	USA	2	1.83 (0.94–3.56)
	Vleming et al., 1998 (37)	EDN	Normoalb >15 years of diabetes	96			82	Case–control	Netherlands	1	1.41 (0.97–2.05)
	Vleming et al., 1999 (38)	ADN	Normoalb >15 years of diabetes	79			82	Case–control	Netherlands	1	*
	Wu et al., 2000 (39)	EDN+ADN	Normoalb	27			41	Case–control	China	2	2.60 (1.27–5.32)
	Yoshida et al., 1996 (40)	ADN	Normoalb	72			60	Case–control	Japan	2	1.76 (1.06–2.91)
	Young et al., 1998 (41)	EDN	Normoalb	20			54	Case–control	China (Han)	2	0.96 (0.44–2.09)
AKR1B1											
rs759853	Fanelli et al., 2002 (42)	EDN+ADN	Normoalb	231	126	105	157	Case–control	France	1	0.90 (0.67–1.21)
	Gosek et al., 2005 (43)	EDN	Normoalb	129			162	Case–control	Poland	2	0.96 (0.68–1.34)
	Moczulski et al., 2000 (44)	EDN+ADN	Normoalb ≥15 years of diabetes (without anti-hypertensiva)	221			193	Case–control	USA	1	1.43 (1.07–1.90)
	Neamat-allah et al., 2001 (45)	EDN	Normoalb	85			146	Case–control	England	2	1.56 (1.07–2.29)
		EDN	Normoalb >10 years of diabetes	181			154	Case–control	USA (Pima Indians)	2	1.42 (0.95–2.11)
		EDN	Normoalb >20 years of diabetes	107			102	Case–control	Ireland	1	2.47 (1.61–3.79)
		EDN	Normoalb >15 years of diabetes	77			85	Case–control	England	1	2.15 (1.37–3.37)
	Sivenius et al., 2004 (46)	EDN	Normoalb	4			68	Case–control	Finland	2	2.05 (0.39–10.93)
	So et al., 2008 (47)	ADN	Stable kidney function (8 years follow-up)	208			866	Follow-up	China	2	1.25 (0.97–1.61)
CA-repeat	Chistyakov et al., 1997 (48)	EDN	Normoalb >20 years of diabetes	10			15	Case–control	Russia	1	1.05 (0.28–3.93)
	Dyer et al., 1999 (49)	EDN	Normo-/microalb (long-term)	211			129	Case–control	UK	1	0.93 (0.67–1.28)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
	Fanelli et al., 2002 (42)	EDN+ADN	Normoalb	231	126	105	157	Case–control	France	1	1.04 (0.77–1.40)
	Heesom et al.,1997 (50)	EDN	Normoalb 20 years of diabetes (without complications)	75			43	Case–control	UK	1	1.91 (1.02–3.59)
	Ichikawa et al., 1999 (51)	EDN	Normoalb (no nephropathy)	26			46	Case–control	Japan	2	0.84 (0.40–1.79)
	Lajer et al., 2004 (52)	EDN	Normoalb >15 years of diabetes	431			468	Case–control	Denmark	1	1.11 (0.92–1.35)
	Liu et al., 2002 (53)	EDN	Normoalb	52			128	Case–control	China	2	1.41 (0.80–2.46)
	Maeda et al., 1999 (54)	EDN	Normoalb	63			123	Case–control	Japan	2	0.68 (0.40–1.14)
	Moczulski et al., 2000 (44)	EDN+ADN	Normoalb ≥15 years of diabetes (without antihypertensiva)	221			193	Case–control	USA	1	0.95 (0.63–1.44)
	Moczulski et al., 1999 (55)	EDN	Normoalb	70			179	Case–control	Poland	2	1.52 (1.13–2.03)
	Neamat-allah et al., 2001(45)	EDN	Normoalb	81			137	Case–control	England	2	1.06 (0.71–1.58)
		EDN	Normoalb >10 years of diabetes	174			141	Case–control	USA (Pima Indian)	2	1.25 (0.87–1.79)
		EDN	Normoalb >20 years of diabetes	101			110	Case–control	Ireland	1	0.94 (0.63–1.39)
		EDN	Normoalb >15 years of diabetes	67			78	Case–control	England	1	0.83 (0.51–1.35)
	Ng et al., 2001 (56)	EDN	Normoalb >15 years of diabetes (without complications)	15			49	Case–control	Australia	1	1.19 (0.48–2.92)
	Park et al., 2002 (57)	EDN+Ret	Normoalb >10 years of diabetes (without complications)	48			38	Case–control	Korea	2	1.43 (0.73–2.80)
	So et al., 2008 (47)	ADN	Stable kidney function (8 years follow-up)	208			866	Follow-up	China	2	1.39 (1.09–1.77)
	Yamamoto et al., 2003 (58)	EDN	Normoalb	19			67	Case–control	Japan	1	1.08 (0.40–2.90)
	Zhao et al., 2004 (59)	autopsy proven DN	Normal biopsy	135			51	Case–control	China	2	0.96 (0.59–1.58)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
APOC1											
rs4420638	McKnight et al., 2009 (60)	EDN+Ret	Normoalb >15 years of diabetes (without complications, without anti-hypertensiva)	590			494	Case–control	UK	1	1.53 (1.23–1.90)
		EDN+Ret	Normoalb 15 years (without complications, without anti-hypertensiva)	267			441	Case–control	Ireland	1	1.56 (1.18–2.07)
APOE											
E2, E3, E4	Araki et al., 2000 (61)	EDN+ADN	Normoalb >15 years of diabetes	223			196	Case–control	USA	1	3.74 (2.06–6.79)
	Chowdhury et al., 1998 (62)	EDN	Normo-/microalb (>50 years of diabetes)	252			197	Case–control	UK	1	3.05 (1.22–7.62)
	Eto et al., 1995 (63)	EDN+ADN	Normoalb	100	43	57	135	Case–control	Japan	2	3.08 (1.30–7.30)
	Ha et al., 1999 (64)	EDN	Normoalb (normal renal function)	74			93	Case–control	Korea	2	1.20 (0.62–2.30)
	Hadjadj et al., 2000 (65)	EDN+ADN	Normoalb (normal renal function)	114			77	Case–control	France	1	3.29 (1.57–6.89)
	Horita et al., 1994 (66)	ADN	Normoalb	57			398	Case–control	Japan	2	0.83 (0.31–2.22)
	Kimura et al., 1998 (67)	ADN	Normoalb	81			97	Case–control	Japan	2	0.51 (0.03–8.36)
	Leiva et al., 2007 (68)	ADN	Normoalb (without complications)	56			29	Case–control	Chili	2	3.03 (1.60–5.73)
	Onuma et al., 1996 (69)	EDN+ADN	Normoalb >10 years of diabetes	41			74	Case–control	USA	1	0.76 (0.28–2.05)
	Tarnow et al., 2000 (70)	EDN+no Ret	Normoalb >15 years of diabetes	197			192	Case–control	Denmark	1	1.25 (0.76–2.05)
	Yakunina et al., 2005 (71)	EDN	Normo-/microalb ≥20 years of diabetes	62			68	Case–control	Russia	1	0.66 (0.33–1.30)
CCR5											
rs1799987	Ahluwalia et al., 2009 (72)	EDN	Normoalb ≥10 years of diabetes	240			255	Case–control	North India	2	0.46 (0.35–0.59)
		EDN	Normoalb ≥10 years of diabetes	96			92	Case–control	South India	2	0.46 (0.30–0.70)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
	Mlynarski et al., 2005 (73)	EDN+ADN	Normoalb ≥15 years of diabetes (without antihypertensiva)	496			298	Case–control	USA	1	1.19 (0.97–1.46)
	Nakajima et al., 2003 (74)	EDN	Normoalb	95			355	Case–control	Japan	2	0.85 (0.61–1.17)
	Pettigrew et al., 2010 (75)	EDN+Ret	Normoalb 15 years (without complications, without anti-hypertensiva)	263			437	Case–control	Ireland	1	0.98 (0.79–1.22)
	Prasad et al., 2007 (76)	ADN	Normoalb ≥10 years of diabetes	196			205	Case–control	India	2	0.61 (0.46–0.80)
	Tregouet et al., 2008 (77)	EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	489			463	Case–control	Denmark	1	1.00 (0.83–1.20)
		EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	387			391	Case–control	France	1	1.00 (0.83–1.21)
		EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	300			469	Case–control	Finland	1	1.00 (0.81–1.24)
CNDP1											
D18S880	Freedman et al., 2007 (78)	ADN	No nephropathy	165			258	Case–control	USA	2	0.77 (0.58–1.01)
	Janssen et al., 2005 (79)	EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	114			71	Case–control	Germany	2	0.78 (0.52–1.17)
		EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	21			36	Case–control	Germany	1	0.37 (0.17–0.81)
	Mooyaart et al., 2010 (80)	ADN	Normoalb ≥15 years	65			93	Case–control	Netherlands/ Germany	2	**
	Tregouet et al., 2008 (77)	EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	489			463	Case–control	Denmark	1	0.94 (0.82–1.07)
		EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	387			391	Case–control	France	1	1.01 (0.84–1.21)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
		EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	300			469	Case–control	Finland	1	0.99 (0.82–1.20)
	Wanic et al., 2008 (81)	EDN+ADN	Normoalb ≥15 years of diabetes (without anti-hypertensiva)	656	445	221	613	Case–control	USA	1	1.03 (0.83–1.28)
ELMO1											
rs741301	Shimazaki et al., 2005 (82)	EDN+ADN+Ret	Normoalb	87			92	Case–control	Japan	2	1.84 (1.18–2.86)
		EDN+ADN+Ret	Normoalb	459			242	Case–control	Japan	2	1.51 (1.19–1.91)
	Pezzolesi et al., 2009 (83)	EDN+ADN	Normoalb ≥15 years of diabetes	820			885	Case–control	USA	1	0.88 (0.76–1.01)
EPO											
rs1617640	Tong et al., 2008 (84)	EDN+ADN	Normoalb ≥15 years (without nephropathy or Ret)	374			239	Case–control	USA	2	0.69 (0.55–0.87)
		EDN+ADN	Normoalb ≥15 years (without nephropathy or Ret)	865			574	Case–control	USA	1	0.65 (0.56–0.76)
		EDN+ADN	Normoalb ≥15 years (without nephropathy or Ret)	379			141	Case–control	USA	1	0.72 (0.55–0.95)
GLUT1											
rs841853	Grzeszczak et al., 2001 (85)	EDN	Normoalb	132			162	Case–control	Poland	2	1.82 (1.13–2.93)
	Gutierrez et al., 1998 (86)	EDN	Normoalb	20			100	Case–control	Spain	2	0.57 (0.20–1.60)
	Hodgkinson et al., 2005 (87)	EDN	No complications ≥20 years of diabetes	101			56	Case–control	UK	1	1.17 (0.73–1.86)
	Liu et al., 1999 (88)	EDN	No nephropathy	64			45	Case–control	China (Han)	2	0.52 (0.29–0.93)
	Makni et al., 2008 (89)	EDN	Normoalbuminuria >10 years of diabetes	126			273	Case–control	Tunesia	2	1.05 (0.62–1.79)
	Ng et al., 2002 (90)	EDN+ADN	Normoalb ≥15 years of diabetes	249			207	Case–control	USA	1	0.81 (0.56–1.17)
	Tarnow et al., 2001 (91)	EDN	Normoalb	175			192	Case–control	Denmark	1	0.73 (0.48–1.10)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
GREM 1											
rs1129456	McKnight et al., 2010 (92)	EDN	Normoalb >15 years of diabetes (without complications and without anti-hypertensiva)	264			439	Case–control	Ireland	1	1.73 (1.24–2.41)
		EDN+ADN	Normoalb, ≥15 years of diabetes (without anti-hypertensiva)	595			501	Case–control	UK	1	1.42 (1.09–1.85)
HSPG2											
rs3767140	Fujita et al., 1999 (93)	EDN	Normoalb (without nephropathy)	102			64	Case–control	Japan	2	0.67 (0.43–1.05)
	Hansen et al., 1997 (94)	EDN	Normoalb >20 years	170			90	Case–control	Denmark	1	0.60 (0.39–0.92)
		EDN+Ret	Normoalb >20 years	247			150	Case–control	UK	1	0.68 (0.48–0.96)
	Liu et al., 2003 (95)	EDN+ADN	Normoalb ≥10 years	213	163	50	77	Case–control	China (Han)	2	0.91 (0.63–1.33)
VEGFA											
rs833061	McKnight et al., 2007 (96)	EDN+Ret	Normoalb >15 years of diabetes (without complications and without anti-hypertensiva)	153			184	Case–control	Ireland	1	0.46 (0.34–0.62)
		EDN+Ret	Normoalb 15 years (without complications of diabetes and without anti-hypertensiva)	89			117	Case–control	Ireland	1	0.51 (0.35–0.76)
FRMD3											
rs1888747	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	379 [g]			413 [g]	Case–control	USA	1	1.33 (1.01–1.75)
		EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	441 [h]			472 [h]	Case–control	USA	1	1.47 (1.20–1.80)
		EDN+ADN	Normo-/microalb (diabetes 16–22 years)	132			1172	Follow-up			1.27 (1.02–1.58)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
rs10868025	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years of diabetes (without anti-hypertensiva)	379 [g]			413 [g]	Case–control	USA	1	1.23 (0.96–1.58)
		EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	441 [h]			472 [h]	Case–control	USA	1	1.52 (1.26–1.83)
		EDN+ADN	Normo-/microalb (diabetes 16–22 years)	132			1172	Follow-up			1.35 (1.10–1.66)
CARS											
rs451041	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	379 [g]			413 [g]	Case–control	USA	1	1.32 (1.03–1.69)
		EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	441 [h]			472 [h]	Case–control	USA	1	1.38 (1.15–1.66)
		EDN+ADN	Normo-/microalb (diabetes 16–22 years)	132			1172	Follow-up			1.38 (1.13–1.69)
rs739401	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	379 [g]			413 [g]	Case–control	USA	1	1.38 (1.13–1.68)
		EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	441 [h]			472 [h]	Case–control	USA	1	1.27 (1.06–1.53)
ACACB											
rs2268388	Maeda et al., 2010 (98)	EDN+Ret	Normoalb	737			552	Case–control	Japan	2	1.61 (1.33–1.96)
		ADN+Ret	Normoalb ≥15 years of diabetes (Ret)	177			196	Case–control	Korea	2	0.83 (0.59–1.17)
		EDN	Normoalb >7 years of diabetes	199			212	Case–control	Singapore (Han)	2	1.07 (0.78–1.48)
		EDN	Normoalb	428			425	Case–control	Denmark	1	0.99 (0.76–1.29)
		EDN+Ret	Normoalb >5 years of diabetes	473			415	Case–control	USA	2	1.61 (1.22–2.12)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
ADIPOQ											
rs17300539	Jorsal et al., 2008 (99)	EDN	Normoalb >15 years of diabetes	438			440	Case–control	Denmark	1	1.46 (1.03–2.08)
	Prior et al., 2008 (100)	EDN+Ret	Normoalb ≥50 years of diabetes	98			99	Case–control	UK	1	2.19 (1.16–4.12)
	Zhang et al., 2009 (101)	EDN+ADN	Normoalb ≥15 years of diabetes	578			599	Case–control	USA	1	1.02 (0.78–1.33)
HP											
Hp 1/2	Awadallah et al., 2008 (102)	EDN	Normoalb	37			89	Case–control	Jordan	2	1.47 (0.82–2.62)
	Bessa et al., 2007 (103)	EDN	Normoalb	20			20	Case–control	Egypt	2	0.24 (0.09–0.63)
	Conway et al., 2007 (104)	EDN+Ret	Normoalb ≥15 years (without anti-hypertensiva)	224			285	Case–control	Ireland	1	0.74 (0.57–0.96)
	Costacou et al., 2009 (105)	EDN	Normoalb	62			163	Case–control	USA	1	0.89 (0.58–1.38)
	Moczulski et al., 2001 (106)	EDN+ADN	Normoalb ≥15 years of diabetes	312			290	Case–control	USA	1	1.00 (0.80–1.26)
	Nakhoul et al., 2001 (107)	EDN	Normoalb	5			43	Case–control	Israel	1	0.11 (0.01–0.92)
		EDN	Normoalb	10			38	Case–control	Israel	2	0.31 (0.07–1.46)
	Wobeto et al., 2009 (108)	EDN	Normoalb	48			128	Case–control	Brazil	1, 2	0.96 (0.60–1.55)
PVT 1											
rs11993333	Hanson et al., 2007 (109)	ADN	Normoalb (>10 years of diabetes)	102			103	Case–control	USA (Pima Indians)	2	0.48 (0.31–0.74)
	Millis et al., 2007 (110)	EDN+ADN	Normoalb ≥15 years of diabetes	526			558	Case–control	USA	1	0.82 (0.69–0.97)
CPVL/CHN2											
rs39059	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	379 [g]			413 [g]	Case–control	USA	1	0.70 (0.57–0.87)
		EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	441 [h]			472 [h]	Case–control	USA	1	0.77 (0.64–0.93)
rs39075	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	379 [g]			413 [g]	Case–control	USA	1	1.18 (0.93–1.50)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
		EDN+ADN	Normoalb ≥15 years (without anti-hypertensiva)	441 [h]			472 [h]	Case–control	USA	1	0.75 (0.63–0.90)
		EDN+ADN	Normo-/microalb (diabetes 16–22 years)	132			1172	Follow-up	USA	1	0.68 (0.55–0.84)
AGT											
rs699	Ahluwalia et al., 2009 (1)	EDN+ADN	Normoalb ≥10 years of diabetes	240			255	Case–control	India	2	2.44 (1.89–3.16)
	Chowdhury et al., 1996 (5)	EDN+Ret	Normo-/microalb ≥20 years of diabetes	242			139	Case–control	UK	1	0.76 (0.38–1.51)
	Doria et al., 1996 (111)	EDN	Normoalb ≥15 years of diabetes	100			100	Case–control	USA	1	0.66 (0.31–1.38)
	Fogarty et al., 1996 (112)	EDN	Normoalb >20 years diabetes (without anti-hypertensiva)	95			100	Case–control	Ireland	1	0.81 (0.66–0.99)
	Fradin et al., 2002 (8)	EDN	Normoalb	39			118	Case–control	France	2	1.08 (0.82–1.41)
	Freire et al., 1998 (113)	EDN	Normoalb; 10 years of diabetes	115			118	Case–control	USA	2	0.86 (0.49–1.50)
	Guitterez et al., 1997 (11)	EDN	Normoalb	20			100	Case–control	Spain	2	1.04 (0.84–1.28)
	Marre et al., 1997 (17)	EDN+ADN	Normoalb	233	126	107	157	Case–control	France	1	2.71 (0.88–8.36)
	Miura et al., 1999 (18)	EDN	Normoalb >10 years of diabetes	32			103	Case–control	Japan	1	1.24 (0.92–1.69)
	Mollsten et al., 2008 (19)	EDN+ADN	Normoalb ≥20 years	48			197	Case–control	Sweden	1	1.37 (0.92–2.05)
	Ohno et al., 1996 (24)	EDN	Normoalb	25			53	Case–control	Japan	2	1.21 (0.80–1.83)
	Osawa et al., 2007 (114)	EDN+ADN	Normoalb+Ret	735			551	Case–control	Japan	2	1.05 (0.63–1.77)
	Prasad et al., 2006 (27)	ADN+Ret	Normoalb ≥10 years of diabetes	196			225	Case–control	India	2	1.20 (0.83–1.75)
	Tarnow et al., 1996 (115)	EDN	Normoalb >15 years of diabetes	195			185	Case–control	Denmark	1	1.15 (0.86–1.53)
	Thomas et al., 2001 (33)	EDN	Normoalb	51			255	Case–control	China (Han)	2	0.69 (0.44–1.10)
	Tregouet et al., 2008 (77)	EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	489			463	Case–control	Denmark	1	1.06 (0.88–1.27)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
		EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	387			391	Case–control	France	1	0.87 (0.72–1.06)
		EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	300			469	Case–control	Finland	1	1.22 (0.99–1.51)
	van Ittersum et al., 2000 (35)	EDN	Normoalb	30			188	Case–control	Netherlands	1	0.98 (0.73–1.32)
	Young et al., 1998 (41)	EDN	Normoalb	20			54	Case–control	China (Han)	2	1.61 (0.93–2.79)
	Zychma et al., 2000 (116)	EDN+ADN	Normoalb ≥10 years of diabetes	127			243	Case–control	Poland	2	0.90 (0.67–1.22)
AGTR1											
rs5186	Ahluwalia et al., 2009 (1)	EDN+ADN	Normoalb ≥10 years of diabetes	240			255	Case–control	India	2	0.68 (0.51–0.89)
	Chistyakov et al., 1999 (117)	EDN	Normo-/microalb ≥20 years of diabetes and no macroalbuminuria	27			41	Case–control	Russia	1	0.82 (0.38–1.76)
	Chowdhury et al., 1997 (118)	EDN+Ret	Normo-/microalb ≥20 years of diabetes	264			136	Case–control	UK	1	0.88 (0.64–1.22)
	Fradin et al., 2002 (8)	EDN	Normoalb	39			118	Case–control	France	2	1.78 (0.92–3.45)
	Mollsten et al., 2008 (19)	EDN+ADN	Normoalb ≥20 years of diabetes	48			197	Case–control	Sweden	1	1.14 (0.83–1.56)
	Marre et al., 1997 (17)	EDN+ADN	Normoalb	233	126	107	157	Case–control	France	1	1.55 (0.89–2.72)
	Osawa et al., 2007 (114)	EDN+ADN	Normoalb+Ret	735			551	Case–control	Japan	2	0.87 (0.65–1.16)
	Savage et al., 1999 (119)	EDN	Normoalb >20 years of diabetes	95			97	Case–control	UK	1	1.05 (0.67–1.63)
	Tarnow et al., 1996 (120)	EDN	Normoalb >15 years of diabetes	198			190	Case–control	Denmark	1	0.98 (0.72–1.34)
	Thomas et al., 2001 (33)	EDN	Normoalb	51			255	Case–control	China (Han)	2	1.02 (0.74–1.40)
	van Ittersum et al., 2000 (35)	EDN	Normoalb	30			188	Case–control	Nether-lands	1	1.84 (0.55–6.20)
	Vionnet et al., 2006 (121)	EDN+Ret	Normoalb ≥15 years of diabetes	390			385	Case–control	Den-mark	1	2.10 (1.10–4.01)
		EDN+Ret	Normoalb ≥15 years of diabetes	387			469	Case–control	Finland	1	1.06 (0.85–1.32)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
		EDN+Ret	Normoalb ≥15 years of diabetes	280			273	Case–control	France	1	0.85 (0.67–1.09)
	Young et al., 1998 (41)	EDN	Normoalb	20			54	Case–control	China (Han)	2	1.09 (0.84–1.42)
NOS3											
rs2070744	Ahluwalia et al., 2008 (122)	EDN	Normoalb ≥10 years (without anti-hypertensiva)	195			255	Case–control	India	2	1.27 (0.91–1.76)
	Zanchi et al., 2000 (123)	EDN+ADN	Normoalb ≥15 years of diabetes	152	74	78	195	Case–control	USA	1	1.57 (1.08–2.29)
rs3138808	Ahluwalia et al., 2008 (122)	EDN	Normoalb ≥10 years (without antihypertensiva)	195			255	Case–control	India	2	1.35 (0.94–1.94)
	Fujita et al., 2000 (124)	EDN	Normoalb	65			102	Case–control	Japan	2	0.73 (0.33–1.60)
	Neugebauer et al., 2000 (125)	EDN	Normoalb >5 years of diabetes	39			82	Case–control	Japan	2	3.02 (1.34–6.81)
	Rippin et al., 2003 (126)	EDN	Normoalb ≥50 years of diabetes	464			396	Case–control	UK	1	1.05 (0.80–1.38)
	Shestakova et al., 2006 (28)	EDN	Normoalb >20 years of diabetes	63			66	Case–control	Russia	1	1.97 (1.16–3.36)
	Shimizu et al., 2002 (127)	EDN+ADN	Normoalb ≥10 years of diabetes	230	107	123	203	Case–control	Japan	2	0.99 (0.66–1.48)
	Taniwaki et al., 2001 (31)	EDN+ADN	Normoalb	42	22	20	69	Case–control	Japan	2	0.94 (0.43–2.10)
	Zanchi et al., 2000 (123)	EDN+ADN	Normoalb ≥15 years of diabetes	152	74	78	195	Case–control	USA	1	1.69 (1.14–2.51)
PPARG											
rs1801282	Caramori et al., 2003 (128)	EDN	Normoalb ≥10 years diabetes	104			212	Case–control	Brazil	2	0.50 (0.26–0.97)
	Hermann et al., 2002 (129)	EDN+ADN	Normoalb (without complications)	241	197	44	203	Case–control	Germany	2	0.83 (0.57–1.21)
	Liu et al., 2010 (130)	EDN	Normoalb ≥10 years of diabetes (without antihypertensiva)	532			228	Case–control	Shanghai (China/Han)	2	1.16 (0.90–1.51)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
	Jorsal et al., 2008 (131)	EDN	Normoalb ≥15 years diabetes (geen antihypertensiva)	415			428	Case–control	Denmark	1	0.43 (0.30–0.63)
	Tregouet et al., 2008 (77)	EDN+ADN	Normoalb ≥5 years of diabetes	489			463	Case–control	Denmark	1	1.20 (0.94–1.54)
		EDN+ADN	Normoalb ≥5 years of diabetes	387			391	Case–control	France	1	0.97 (0.76–1.24)
		EDN+ADN	Normoalb ≥5 years of diabetes	300			469	Case–control	Finland	1	0.87 (0.61–1.25)
UNC13B											
rs13293564	Tregouet et al., 2008 (77)	EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	484	445	39	459	Case–control	Denmark	1	1.27 (1.05–1.53)
		EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	290	226	64	370	Case–control	France	1	1.05 (0.84–1.31)
		EDN+ADN	Normoalb ≥5 years of diabetes (without anti-hypertensiva)	386	264	122	467	Case–control	Finland	1	1.30 (1.07–1.58)
		EDN+ADN	Normoalb	412	269	143	314		Finland	1	1.25 (1.04–1.49)
No gene											
rs1041466	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years of diabetes	379 [g]			413 [g]	Case–control	USA	1	1.22 (0.52–2.86)
		EDN+ADN	Normoalb ≥15 years of diabetes	441 [h]			472 [h]	Case–control	USA	1	1.38 (1.15–1.66)
		EDN+ADN	Normo-/microalb (diabetes 16–22 years)	132			1172	Follow-up	USA	1	1.39 (1.14–1.69)
rs1411766	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years of diabetes	379 [g]			413 [g]	Case–control	USA	1	0.85 (0.66–1.10)
		EDN+ADN	Normoalb ≥15 years of diabetes	441 [h]			472 [h]	Case–control	USA	1	0.69 (0.57–0.83)
		EDN+ADN	Normo-/microalb (diabetes 16–22 years)	132			1172	Follow-up	USA	1	0.75 (0.62–0.91)

Variants	Article	Definitions		Cases of DN or controls (*n*)				Study details			OR[a]
		Cases	Controls	Total	EDN[b]	ADN[c]	Controls	Design	Country	TD	Allele level
rs79899848	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years	379 [g]			413 [g]	Case–control	USA	1	0.93 (0.72–1.20)
		EDN+ADN	Normoalb ≥15 years of diabetes	441 [h]			472 [h]	Case–control	USA	1	1.33 (1.10–1.61)
		EDN+ADN	Normo-/microalb (diabetes 16–22 years)	132			1172	Follow-up	USA	1	1.32 (1.08–1.61)
rs9521445	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years of diabetes	379 [g]			413 [g]	Case–control	USA	1	1.32 (1.09–1.61)
		EDN+ADN	Normoalb ≥15 years of diabetes	441 [h]			472 [h]	Case–control	USA	1	1.38 (1.14–1.65)
rs6492208	Pezzolezi et al., 2009 (97)	EDN+ADN	Normoalb ≥15 years of diabetes	379 [g]			413 [g]	Case–control	USA	1	0.90 (0.70–1.16)
		EDN+ADN	Normoalb ≥15 years of diabetes	441 [h]			472 [h]	Case–control	USA	1	1.33 (1.10–1.61)
		EDN+ADN	Normo-/microalb (diabetes 16–22 years)	132			1172	Follow-up	USA	1	1.32 (1.08–1.61)

* Vleming 1999 and Vleming 1998 are considered as one dataset, as they use the same control group

** Mooyaart 2010 and Janssen 2005 are considered as one dataset, as they use the same control group

[a]OR (95% CI)

[b]EDN, established diabetic nephropathy; [c]ADN, advanced diabetic nephropathy; [d]Normoalb, normoalbuminuria; [e]microalb, microalbuminuia; [f]Ret, retinopathy; [g]GWU, George Washington University; [h]JDC, Joslin Diabetes Center

DN, diabetic nephropathy; TD, type of diabetes

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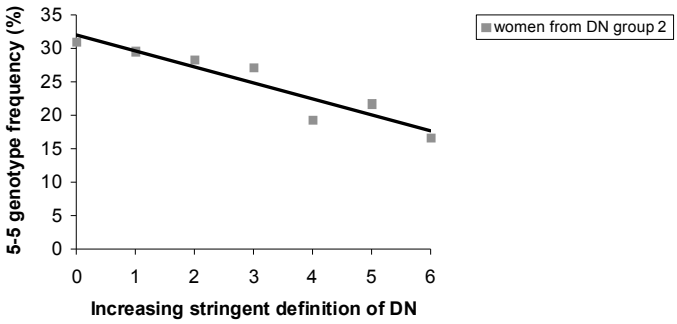
Hardy-Weinberg equilibrium

	Population		Type 2 diabetes population		DN group 1		DN group 2		DN group 3		Diabetic non-nephropathy controls	
	N = 472		N = 562		N = 114		N = 90		N = 66		N = 93	
CNDP1 genotype	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
5-5	197	201.0	214	205.7	32	35.4	32	31.2	27	25.2	40	3508
5-6	194	186.0	230	243.8	58	52.3	39	38.9	23	27.4	34	52.8
5-7	28	28.1	22	24.8	4	2.8	3	4.7	4	3.1	1	208
6-6	40	43.0	79	72.7	17	19.3	11	12.1	10	7.4	15	19.3
6-7	11	13.0	15	14.7	1	2.1	5	2.9	1	1.7	1	2.1
7-7	2	1.0	2	0.7	0	0.1	0	0.2	0	0.1	0	0.1
P	0.57		0.24		0.50		0.50		0.50		0.43	

Sensitivity analysis



	Increasing stringent definition of DN:	women (n)	5-5 homozygous frequency (%)
0	MDRD < 60	220	31
1	MDRD < 60 and age < 70	88	30
2	DN as in manuscript	60	28
3	MDRD < 60 + microalbuminuria	59	27
4	MDRD < 45	52	19
5	MDRD < 45 + microalbuminuria	23	22
6	MDRD < 30	6	17

With increasing stringent definition of the diagnosis diabetic nephropathy the frequency of the 5-5 homozygous genotype decreases in women, suggesting that the protective effect will only be stronger with a more stringent definition of diabetic nephropathy.

Permutation studies

We first analyzed Hardy-Weinberg Equilibrium (HWE) for the total dataset and various subgroups (table 1) to see if there would be indications for population stratification. Stratification by sex and disease status does not reveal any deviation from HWE. Hence, HWE analysis does not give an indication on population strata.

Table 1. Tests for deviation from HWE in several subgroups of the data set. Subgroups are characterized by *Sex* (F=female, M=male) and disease status. P-values are given for the Chi-Square goodness of fit test. *N* denotes the sample size in the subgroups.

Sex	Disease status	p-value	N
F	no DN	0.53	44
M	no DN	0.90	47
Both	no DN	0.43	91
F	DN	0.56	139
M	DN	0.74	128
Both	DN	0.95	267
F	All	0.92	183
M	All	0.48	175
Both	All	0.85	358

As individual ethnicity is not known for all patients in this sample and the sample is in almost perfect HWE, it is difficult to construct a permutation scheme that incorporates population strata. We therefore first performed a permutation test without incorporating population strata by randomly permuting phenotype status across the whole data set. Such a procedure can primarily account for small sample size. The permuted P-values are lower than P-values based on the asymptotic Chi-Square distribution (table 2), indicating that small sample size cannot explain the P-values in our study. The asymptotic P-values behave conservative in this situation.

Table 2. P-values for genetic association of the 5-5 genotype in a recessive model. Column *Total* lists P-values for the combined sample (all cases are treated as a single group).

	Total	DN group 1	DN group 2	DN group 3
Asymptotic P-value	0.0000358	0.000542	0.00102	0.00689
Permuted P-value	0.0000073	0.000234	0.000444	0.00281

Sensitivity analysis of population stratification

We addressed the question of population stratification by a sensitivity analysis. The sensitivity analysis was performed in R version 2.10.0. For all Chi-Square tests a continuity correction was used leading to slightly different numeric results compared to the paper. The sensitivity analysis is based on the so-called inflation factor used in genome wide association studies (Biometrics, 55. p.997-1004, 1999), which assesses how much the average/median test statistic of single nucleotide polymorphisms, based on a Chi-Square distribution with one degree of freedom, deviates from the expectation. If the inflation factor is greater than 1, there is an indication that there might be population stratification. This inflation factor can be used to correct results from genome wide association studies by dividing the test statistic by the inflation factor, thereby assuring that a re-analysis is uninflated. We used this concept to determine how large the inflation factor could be in our study to still get significant results at a certain significance level (table 3).

For all groups an inflation of 1.1 is allowed to still achieve a significance of 0.01. An inflation factor of 1.1 is larger than the maximal inflation factor observed in the WTCCC study (Nature, 447. p.661-678, 2007). The maximal reported inflation factor for a genome wide association studies is 1.4 to our knowledge (BMC Proc, 3 Suppl 7. s.13, 2009) (NARAC study). Note, that group 2 and group 3 do not reach the significance

level of 10^{-3} but the combined sample is still significant and still exceeds the inflation factor 1.4 from the NARAC study. We have repeated the analysis with a permutation test in the individual groups and present these results in table 4.

In conclusion, there is no indication for a systematic error due to population stratification based on our sensitivity analysis.

Table 3. Sensitivity analysis for P-values of the study. For an assumed inflation factor the significance level would be precisely alpha for inflation factor > 1. For inflation factor = 1 the nominal p-value is greater than alpha.

	P-value	Alpha	Inflation factor
DN group 1	0.0005	0.050	3.11
DN group 2	0.0010	0.050	2.81
DN group 3	0.0070	0.050	1.89
All	<0.0001	0.050	4.45
DN group 1	0.0005	0.010	1.80
DN group 2	0.0010	0.010	1.63
DN group 3	0.0070	0.010	1.10
All	<0.0001	0.010	2.57
DN group 1	0.0005	0.001	1.10
DN group 2	0.0010	0.001	1.00
DN group 3	0.0070	0.001	1.00
All	<0.0001	0.001	1.58

Table 4. Sensitivity analysis for P-values using a permutation test. For an assumed inflation factor the significance level would be precisely alpha for inflation factor > 1. For inflation factor = 1 the nominal p-value is greater than alpha.

	P-value	Alpha	Inflation factor
DN group 1	0.0004	0.050	3.31
DN group 2	0.0070	0.050	3.03
DN group 3	0.0045	0.050	2.10
All	<0.0001	0.050	4.53
DN group 1	0.0004	0.010	1.92
DN group 2	0.0070	0.010	1.75
DN group 3	0.0045	0.010	1.22
All	<0.0001	0.010	2.63
DN group 1	0.0004	0.001	1.17
DN group 2	0.0070	0.001	1.07
DN group 3	0.0045	0.001	1.00
All	<0.0001	0.001	1.61

A

B

List of abbreviations

B

ACE	Angiotensin-converting enzyme
AGE	Advanced glycation end products
CD-CV model	Common disease-common variant model
DN	Diabetic nephropathy
EM	Electron microscopy
ESRD	End stage renal disease
GBM	Glomerular basement membrane
GWAS	Genome wide association scan
Hindinef	Hindustani Diabetic Nephropathy Study
HWE	Hardy-Weinberg equilibrium
IFTA	Interstitial fibrosis and tubular atrophy
MODY	Maturity onset diabetes of the young
NECOSAD	Netherlands Cooperative Study on the Adequacy of Dialysis
RAME model	Rare alleles of major effect model
SNP	Single nucleotide polymorphism
SUNSET	Surinamese in the Netherlands, study for ethnicity and health
ZODIAC	Zwolle Outpatient Diabetes Project Integrating Available Care

B

C

Curriculum Vitae

C

Antien Mooyaart werd op 30 augustus 1986 geboren in Gouda en groeide daar op in een gezin van drie kinderen, waarvan zij de middelste was. Na het behalen van haar eindexamen op het Coornhert-gymnasium in Gouda, ging zij in 2004 in Leiden, de stad van haar keuze, geneeskunde studeren en werd lid van Minerva. Zij volgde door deze keuze ook de weg van haar grootmoeder, die eveneens in Leiden geneeskunde studeerde, lid was van de Vereniging van Vrouwelijke Studenten te Leiden (VVSL) en later aldaar in 1957 promoveerde. Antien's studententijd kenmerkte zich verder door hockey, huis- en jaarclubactiviteiten en LPC-kampen, internationale zomerkampen voor jongeren, waarin zij als staflid participeerde.

In haar tweede jaar werd ze toegelaten tot het excellente studententraject, waaruit een onderzoektraject op de afdeling Pathologie bij prof. dr J.A. Bruijn is voortgekomen. Dit onderzoek leidde tot een PhD traject bij de onderzoeksgroep nierpathologie, waarmee zij na het behalen van haar doctoraal examen in april 2008 aanving.

In het kader hiervan bezocht ze diverse congressen in binnen- en buitenland, waar ze posters presenteerde en presentaties gaf. Verder volgde zij verschillende cursussen op het gebied van de nierpathologie, de genetica en de epidemiologie, leidend tot haar registratie als klinisch epidemioloog. Op 7 november 2008 kreeg zij de Conrad Pirani Travel Award uitgereikt tijdens de Renal Week van de American Society of Nephrology in Philadelphia.

In juli 2009 is Antien begonnen met haar coschappen en zij hoopt in mei 2012 het artsexamen te behalen.

D

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Als tweedejaars geneeskunde student solliciteerde ik bij **Prof. dr Jan Anthonie Bruijn** voor een onderzoeksplek op de afdeling Pathologie, waarna ik koos voor zijn eigen onderzoekslijn Nierpathologie. Zijn uitgebreide wetenschappelijke ervaring, zijn positieve instelling en niet aflatende vertrouwen in mij zijn altijd een grote steun geweest.

Ik ben onder de hoede gekomen van mijn toekomstige copromotor **Dr Hans Baelde**, die mij in eerste instantie de basale laboratoriumtechnieken heeft bijgebracht. Gedurende mijn gehele promotietraject is hij mijn steun en toeverlaat geweest voor technische, inhoudelijke, wetenschappelijke en praktische vragen.

Mijn andere copromotor **Dr Emile de Heer** wil ik bedanken voor zijn inhoudelijke en wetenschappelijke bijdrage voor de totstandkoming van dit proefschrift. Zijn internationale contacten, kennis en enthousiasme zijn van onschatbare waarde geweest voor dit proefschrift.

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Van mijn co-auteurs wil ik met name **Ingeborg Bajema**, **Olaf Dekkers**, **Diana Grootendorst** en **Irene van Valkengoed** bedanken voor hun bijdrage aan de totstandkoming van dit proefschrift en aan mijn wetenschappelijke ontwikkeling. Verder wil ik graag de bibliothecaris **Jan Schoones** bedanken.

Tijdens mijn promotietraject heb ik twee studenten begeleid, **Ana Zutinic** en **Lisette Valk**, die mij met inzet en enthousiasme hebben geholpen bij het tot stand komen van meerdere hoofdstukken.

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Als laatste wil ik ook graag mijn vrienden en familie bedanken voor hun onuitputtelijke steun.

E

List of publications

Mooyaart AL, van Valkengoed IG, Chandie Shaw PK, Peters V, Baelde HJ, Rabelink TJ, Bruijn JA, Stronks K, de Heer E: Lower frequency of the 5/5 homozygous *CNDP1* genotype in South Asian Surinamese. Diabetes Res.Clin Pract. Sep;85(3):272-8. 2009.

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